AIE-active luminogens as highly efficient free-radical ROS photogenerator for image-guided photodynamic therapy

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Catalog

1. Experimental section .................................................................2
2. Normalized fluorescence spectrum of TCM-CPS in water .................................................................7
3. TEM images of TCM-CP and TCM-CPS in water ..............................................................................8
4. Zeta potential distribution of TCM-CP and TCM-CPS in water ..........................................................8
5. Total ROS generation of TCM-CPS, TCM-CP, RB and Ce6 ..............................................................9
6. Calculated energy of the singlet and triplet excited states .................................................................9
7. Singlet oxygen generation of RB and TCM-CPS ..............................................................................10
8. Free radical generation of RB .........................................................................................................10
9. Comparison of DHR123’s fluorescence intensity with or without Vc .............................................11
10. Hydroxyl radical generation of TCM-CPS .......................................................................................11
11. Confocal images of cells with different incubation time ................................................................12
12. In vitro cytotoxicity of TCM-CPS under hypoxia ..........................................................................12
1. Experimental section

1.1 Materials and general methods

All solvents and starting reactants were purchased from commercial suppliers in analytical grade and used without purification unless special noted. All reagents were weighed by analytical balance (METTLER TOLEDO, ME204E/02). The NMR spectra (\(^1\)H, \(^{13}\)C, and \(^1\)H-\(^1\)H COSY) were obtained from Bruker AM 400 spectrometer, using TMS (\(\delta = 0\)) as internal standard. Waters LCT premier XE spectrometer was used to obtain high resolution mass spectrometry (HRMS) data of the products. UV-Vis absorption spectra and fluorescence spectra were obtained from Agilent Cary 60 UV-Vis spectrophotometer and Agilent Cary Eclipse fluorescence spectrophotometer, respectively. Dynamic light scattering (DLS) experiments were performed on Zetasizer Nano ZSE. The viscosity of solution was measured by TA Instruments DISCOVERY HR-2 Hybrid Rheometer. Transmission electron microscopy (TEM) images were taken on JEOL JEM-1400 instrument. The electron paramagnetic resonance (EPR) signal was obtained with Bruker EMX-8/2.7. Scanning electron microscope images of bacteria were taken on ZEISS GeminiSEM 500 and Hitachi S-3400N. Cell imaging was performed on Lecia TCS SP8 laser scanning confocal microscopy.

1.2 Synthesis route of TCM-CP and TCM-CPS

The important intermediate TCM was synthesized according to our previous work.\(^1\)

Scheme S1. The synthetic route of TCM-CP and TCM-CPS.
1.3 Synthesis of Cz-T-CHO

\[
\begin{align*}
\text{Cz-T-CHO} \quad \text{Cz-T-CHO} \\
\text{N} & \quad \text{N} \\
\text{S} & \quad \text{S} \\
\text{CHO} & \quad \text{CHO} \\
\text{CuI, K_2CO_3} & \quad \text{DMF} \\
\end{align*}
\]

Carbazole (5000 mg, 29.90 mmol), 5-bromo-2-thiophenecarbaldehyde (6858 mg, 35.90 mmol), cuprous iodide (570 mg, 2.99 mmol), anhydrous potassium carbonate (6199 mg, 44.85 mmol) and dry DMF (25 mL) were added to a dry 100 mL three-necked flask in sequence. The mixture was heated at 140 °C under nitrogen atmosphere for 24 h. After cooling to room temperature, the mixture was poured into 100 mL sodium chloride aqueous solution, and the crude product was obtained by extracting three times with dichloromethane (100 mL*3). The organic phase was dried with anhydrous sodium sulfate. After removing the solvent under reduced pressure, the crude product was further purified by silica gel chromatography with petroleum ether/dichloromethane (v/v, 5:1) to afford faint yellow solid (3520 mg, 42% yield). \(^1^H\) NMR (400 MHz, CDCl\(_3\), ppm), \(\delta\): 9.95 (s, 1H, -CHO), 8.11 (d, 2H, \(J = 7.7\) Hz, Ar-H), 7.88 (d, 1H, \(J = 4.0\) Hz, thiophene-H), 7.67 (d, 2H, \(J = 8.2\) Hz, Ar-H), 7.51-7.44 (m, 2H, Ar-H), 7.39-7.31 (m, 3H, Ar-H). \(^{13}\)C NMR (100 MHz, CDCl\(_3\), ppm), \(\delta\): 182.84, 148.63, 140.55, 139.71, 136.10, 126.71, 124.25, 123.67, 121.71, 120.46, 110.34. Mass spectrometry (ESI positive ion mode for [M+H]^+): Calcd. for C\(_{17}\)H\(_{12}\)NOS: 278.0640; found: 278.0616.

1.4 Synthesis of TCM-Cz

\[
\begin{align*}
\text{TCM} & \quad \text{TCM} \\
\text{N} & \quad \text{N} \\
\text{CN} & \quad \text{CN} \\
\text{NC} & \quad \text{NC} \\
\end{align*}
\]

Piperidine (0.25 mL) was added dropwise to 50 mL two-necked flask containing the mixture of TCM (1215 mg, 3.24 mmol), Cz-T-CHO (300 mg, 1.08 mmol) and acetonitrile (15 mL), followed by refluxing at 95 °C under nitrogen atmosphere for 12 h. The mixture was cooled to room temperature, and the solvent was removed under reduced pressure. The solid was further purified by silica gel chromatography with petroleum ether/dichloromethane (v/v, 1:4) to afford red solid (302 mg, 44%
yield). $^1$H NMR (400 MHz, DMSO-$d_6$, ppm), $\delta$: 8.23 (d, 2H, $J = 7.7$ Hz, carbazole-H), 7.73-7.55 (m, 8H, Ar-H), 7.55-7.43 (m, 6H, Ar-H), 7.41 (s, 2H, Ar-H), 7.37-7.29 (m, 2H, Ar-H), 7.21 (s, 1H, Ar-H), 7.11-6.98 (m, 2H, Ar-H), 5.88 (d, 1H, $J = 15.8$ Hz, alkene-H), 2.14 (s, 3H, $-\text{C}_3\text{H}_3$).

$^{13}$C NMR (100 MHz, DMSO-$d_6$, ppm), $\delta$: 166.22, 152.60, 151.06, 147.87, 140.49, 139.56, 137.57, 137.38, 136.47, 131.35, 130.82, 130.70, 130.62, 130.47, 130.28, 129.27, 127.25, 126.76, 126.22, 123.08, 121.25, 120.59, 119.22, 118.64, 118.12, 116.45, 115.09, 110.02, 79.99, 61.25, 21.37. Mass spectrometry (ESI positive ion mode for [M+H]$^+$): Calcd. for C$_{42}$H$_{28}$N$_5$S: 634.2065; found: 634.2061.

1.5 Synthesis of TCM-CP

![Structural Diagram]

Piperidine (0.25 mL) was added dropwise to 50 mL two-necked flask containing the mixture of TCM-Cz (200 mg, 0.32 mmol), 4-pyridinecarboxaldehyde (684 mg, 6.39 mmol) and acetonitrile (15 mL), followed by refluxing at 95 °C under nitrogen atmosphere for 12 h. The mixture was cooled to room temperature, and the solvent was removed under reduced pressure. The solid was further purified by silica gel chromatography with dichloromethane/methanol (v/v, 100:1) to afford red solid (112 mg, 49% yield). $^1$H NMR (400 MHz, DMSO-$d_6$, ppm), $\delta$: 8.54 (d, 2H, $J = 6.0$ Hz, pyridine-H), 8.22 (d, 2H, $J = 7.7$ Hz, carbazole-H), 7.78-7.70 (m, 1H, Ar-H), 7.70-7.57 (m, 7H, Ar-H), 7.54-7.43 (m, 7H, Ar-H), 7.41 (d, 1H, $J = 3.9$ Hz, thiophene-H), 7.38-7.30 (m, 3H, Ar-H), 7.28 (s, 1H, Ar-H), 7.23 (d, 2H, $J = 6.0$ Hz, pyridine-H), 7.19 (s, 1H, Ar-H), 6.97 (d, 1H, $J = 15.9$ Hz, alkene-H), 6.56 (d, 1H, $J = 16.0$ Hz, alkene-H), 5.97 (d, 1H, $J = 15.8$ Hz, alkene-H). $^{13}$C NMR (100 MHz, DMSO-$d_6$, ppm), $\delta$: 166.37, 152.53, 150.41, 148.32, 147.88, 141.55, 140.47, 139.74, 137.43, 136.98, 136.21, 135.44, 131.50, 131.46, 130.96, 130.52, 130.36, 129.43, 127.84, 126.77, 126.17, 124.02, 123.09, 121.27, 121.21, 120.60, 118.94, 117.91, 116.22, 115.52, 110.04, 80.51, 62.32. Mass spectrometry (ESI positive ion mode for [M+H]$^+$): Calcd. for C$_{48}$H$_{31}$N$_6$S: 723.2331; found: 723.2330.
1.6 Synthesis of TCM-CPS

CH$_3$I (80 mg, 0.56 mmol) was added dropwise to 50 mL two-necked flask containing the mixture of TCM-CP (30 mg, 0.04 mmol), and dichloromethane (6 mL), followed by stirring at room temperature for 24 h. The solvent was removed under reduced pressure, and the solid was further purified by silica gel chromatography with dichloromethane/methanol (v/v, 15:1) to afford black solid TCM-CPI (23 mg, 64% yield).

The pure intermediate compound TCM-CPI (21 mg, 0.02 mmol) was dissolved in 5 mL acetone, and 5 mL saturated KPF$_6$ aqueous solution was poured into the acetone solution. The mixture was stirred at room temperature for 12 h, and solids were precipitated out of the solution. After filtering and washing with n-hexane, the black green solid (14 mg) was obtained with the yield of 65%. $^1$H NMR (400 MHz, DMSO-$d_6$, ppm), $\delta$: 8.85 (d, 2H, $J = 5.3$ Hz, pyridinium salt-H), 8.24 (d, 2H, $J = 7.2$ Hz, carbazole-H), 8.03 (d, 2H, $J = 5.2$ Hz, pyridinium salt-H), 7.76-7.59 (m, 8H, Ar-H), 7.57-7.51 (m, 2H, Ar-H), 7.51-7.41 (m, 6H, Ar-H), 7.39-7.21 (m, 6H, Ar-H and alkene-H), 7.01 (d, 1H, $J = 16.0$ Hz, alkene-H), 5.97 (d, 1H, alkene-H, $J = 15.7$ Hz), 4.24 (s, 3H, -CH$_3$). $^{13}$C NMR (100 MHz, DMSO-$d_6$, ppm), $\delta$: 166.47, 152.47, 149.35, 148.42, 147.27, 145.79, 140.45, 139.85, 137.37, 136.82, 136.15, 132.18, 131.68, 131.60, 131.09, 130.52, 130.36, 129.41, 127.94, 126.79, 126.27, 124.74, 123.10, 121.31, 120.64, 119.01, 117.74, 117.03, 116.16, 115.88, 110.04, 80.72, 63.04, 47.41. $^{19}$F NMR (376 MHz, DMSO-$d_6$, ppm), $\delta$: -70.12 ($J = 711$ Hz). Mass spectrometry (ESI positive ion mode for [M-PF$_6$]$^+$): Calcd. for C$_{49}$H$_{33}$N$_6$S: 737.2487; found: 737.2485.

1.7 Transmission electron microscopy imaging of TCM-CP and TCM-CPS

10 µL TCM-CP or TCM-CPS (10 µM in 99% water) was added onto a carbon-coated copper grid, followed by drying overnight at room temperature. The TEM imaging was performed on JEOL JEM-1400 with an accelerating bias voltage of 100 kV.
1.8 Cell culture

Human epithelioid cervical carcinoma (HeLa) cells were purchased from the Institute of Cell Biology (Shanghai, China). Cells were propagated in cell culture flask at 37 °C under humidified 5% CO₂ atmosphere. Dulbecco's modified eagle medium (DMEM, GIBCO/Invitrogen, Camarillo, CA, USA) was supplemented with 1% penicillin-streptomycin (10,000 U mL⁻¹ penicillin, and 10 mg mL⁻¹ streptomycin, Solarbio life science, Beijing, China) and 10% fetal bovine serum (FBS, Biological Industry, Kibbutz Beit Haemek, Israel).

1.9 Intracellular ROS Detection

The HeLa cells were seeded onto glass bottom cell culture dish (Φ 20 mm, NEST). After reaching 80% confluence, the cells were incubated with TCM-CPS (10 μM) for 6 h, followed by incubating with dichlorofluorescein diacetate (DCFH-DA, 20 μM) for 20 min. After light irradiation (40 mW cm⁻², 400-650 nm) for 5 min or in the dark, the cells were imaging by Leica TCS SP8. The PSs+light+vitamin C group: After incubation with TCM-CPS, the cells were treating with DCFH-DA/vitamin C mixtures (50 mM for vitamin C) for 20 min, then irradiated by light before imaging. Green channel: λ<sub>ex</sub> = 488 nm, λ<sub>em</sub> = 505-560 nm.

1.10 Cell ablation study

The viabilities of HeLa cells were evaluated by methylthiazolyldiphenyltetrazolium bromide (MTT) assays. Briefly, the cells were seeded onto 96-well plates at the density of 8×10³ cells per well and cultured for 12 h. The cells were divided into three groups: in the dark, light irradiation for 7.5 min, and light irradiation for 15 min. Then, the cells were incubated with various concentration of TCM-CPS for 6 h, followed by replacing the TCM-CPS solution with fresh DMEM. After light irradiation (40 mW cm⁻²) for different time (7.5 min and 15 min) or in the dark, the cells were further cultured for 12 h. 10 μL MTT solution (5 mg mL⁻¹) was added into each well, and further cultured for 4 h. After removing liquid supernatant, 100 μL DMSO was added into each well to dissolve the crystals. The cell viability was accessed by means of MTT absorbance at 600 nm recorded by a microplate reader (BioTek Synergy H4), and cells incubated with culture medium only in each group were designated as cells with 100% cell viability.

1.11 Live/dead cell staining

The cells were seed onto glass bottom cell culture dish (Φ 20 mm, NEST). After reaching 80% confluence, the cells were incubated with TCM-CPS (10 μM) or fresh DMEM for 6 h. The selected
cells were irradiated by white light (40 mW cm$^{-2}$, 400-650 nm) for 15 min, and further cultured for 12 h. The cells was stained with Calcein-AM/PI mixtures (2 μM for Calcein-AM, 4.5 μM for PI), followed by imaging with Leica TCS SP8. Green channel from Calcein: $\lambda_{\text{ex}} = 488$ nm, $\lambda_{\text{em}} = 495$-540 nm, red channel from PI: $\lambda_{\text{ex}} = 561$ nm, $\lambda_{\text{em}} = 590$-650 nm.

1.12 Bacterial culture and staining

A single colony of bacteria was incubated with LB medium in shaking table (37 °C, 200 r/min). Briefly, the bacteria with an optical density (OD) 0.5 at 600 nm were diluted 100 times in PBS, and incubated with TCM-CMS (10 μM) for various time (15 min, 30 min, and 60 min) in shaking table (37 °C, 200 r min$^{-1}$). After incubation, the bacteria were washed with PBS, and concentrated by centrifuge. Then, the bacteria solution (10 μL) was added onto a glass slide with a coverslip, and imaged by confocal laser scanning microscope (Lecia TCS SP8). Red channel from TCM-CPS: $\lambda_{\text{ex}} = 514$ nm, $\lambda_{\text{em}} = 650$-750 nm.

1.13 Killing the bacteria

The antibacterial activity of TCM-CPS was determined by plate count method. Briefly, the bacterial suspensions were incubated with TCM-CMS (2.5, 5, and 10 μM) for 30 min in shaking table (37 °C, 200 r/min). Then, the bacterial suspensions were either exposed to light (40 mW cm$^{-2}$) for 15 min or under the dark. The bacterial suspensions (30 μL) were transferred onto an agar plate (1.5% agar + LB medium), and further cultured for 12 h, followed by imaging.

2. Normalized fluorescence spectrum of TCM-CPS in water

![Normalized fluorescence spectrum of TCM-CPS in water](image)

**Figure S1** Normalized fluorescence intensity of TCM-CPS in 99% water, $\lambda_{\text{ex}} = 520$ nm.
3. TEM images of TCM-CP and TCM-CPS in water

![TEM images of TCM-CP and TCM-CPS in water](image)

**Figure S2** TEM images of (A) TCM-CP and (B) TCM-CPS in 99% water. All images share scale bar of 100 nm.

4. Zeta potential distribution of TCM-CP and TCM-CPS in water

![Zeta potential distribution](image)

**Figure S3** Zeta potential distribution of TCM-CP and TCM-CPS in 99% water.
5. Total ROS generation of TCM-CPS, TCM-CP, RB and Ce6

Figure S4 Total ROS generation of (A) TCM-CPS, (B) TCM-CP, (C) RB, and (D) Ce6 (10 μM) upon white light irradiation using DCFH (40 μM) as an indicator. (E) The change of solution’ fluorescence without photosensitizer upon white light irradiation. \( \lambda_{ex} = 488 \text{ nm} \).

6. Calculated energy of the singlet and triplet excited states

Table S1 Calculated energy of the singlet (S) and triplet (T) excited states (Gaussian/B3LYP/6-311G(d))

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7. Singlet oxygen generation of RB and TCM-CPS

**Figure S5** Singlet oxygen generation of (A) RB and (B) TCM-CPS (10 μM) upon light irradiation using SOSG (10 μM) as an indicator. (C) The change of solution’ fluorescence without photosensitizer upon white light irradiation. $\lambda_{\text{ex}} = 500$ nm.

8. Free radical generation of RB

**Figure S6** Fluorescence spectra of the DHR123 probe for detecting free-radical ROS generated by RB in the presence of Vc (100 μM) under light irradiation for 10 min. $\lambda_{\text{ex}} = 495$ nm. The concentration of RB is 10 μM.
9. Comparison of DHR123’s fluorescence intensity with or without Vc

![Figure S7](image1.png)

**Figure S7** Comparison of DHR123’s fluorescence intensity with or without Vc.

10. Hydroxyl radical generation of TCM-CPS

![Figure S8](image2.png)

**Figure S8** (A) The hydroxyl radical generation of TCM-CPS (10 μM) upon white light irradiation (80 mW cm\(^{-2}\), 400-650 nm) using HPF (10 μM) as an indicator. \(I_0\) is the fluorescence of mixtures before light irradiation. The change of fluorescence spectra of mixtures (B) with or (C) without TCM-CPS as photosensitizer upon white light irradiation. \(\lambda_{ex} = 460\) nm.
11. Confocal images of cells with different incubation time

Figure S9 Confocal images of HeLa cells incubated with 10 μM TCM-CPS for different time, followed by incubated with 5 μg mL⁻¹ Hoechst 33342 for 15 min. Red channel from TCM-CPS: λ_ex = 514 nm, λ_em = 630-750 nm, blue channel from Hoechst 33342: λ_ex = 405 nm, λ_em = 415-460 nm. Scale bar: 20 μm.

12. In vitro cytotoxicity of TCM-CPS under hypoxia

Figure S10 In vitro cytotoxicity of TCM-CPS under extreme hypoxia environment (Oxygen concentration < 1%). The cells were seeded onto 96-well plate at the density of 8×10³ cells per well and cultured for 12 h. Then, the cells were incubated with various concentration of TCM-CPS for 6 h, followed by replacing the TCM-CPS solution with fresh DMEM. The 96-well plate was put into sealed bag (Hopebio), the oxygen in the bag was consumed by anaerobic gas producing bag (Hopebio) for 2 h. After light irradiation (40 mW cm⁻²) for 15 min, the cells were further cultured
for 12 h. 10 μL MTT solution (5 mg mL⁻¹) was added into each well, and further cultured for 4 h. After removing liquid supernatant, 100 μL DMSO was added into each well to dissolve the crystals. The cell viability was accessed by means of MTT absorbance at 600 nm recorded by a microplate reader (BioTek Synergy H4), and cells incubated with culture medium only were designated as cells with 100% cell viability.
Figure S11 $^1$H NMR spectrum of Cz-T-CHO in CDCl$_3$.

Figure S12 $^{13}$C NMR spectrum of Cz-T-CHO in CDCl$_3$. 
Figure S13 HRMS spectrum of Cz-T-CHO.

Figure S14 $^1$H NMR spectrum of TCM-Cz in DMSO-$d_6$. 
Figure S15 $^{13}$C NMR spectrum of TCM-Cz in DMSO-$d_6$.

Figure S16 HRMS spectrum of TCM-Cz.
Figure S17 $^1$H NMR spectrum of TCM-CP in DMSO-$d_6$.

Figure S18 $^{13}$C NMR spectrum of TCM-CP in DMSO-$d_6$. 
Figure S19 HRMS spectrum of TCM-CP.

Figure S20 $^1$H NMR spectrum of TCM-CPS in DMSO-$d_6$. 
Figure S21 Two-dimensional $^1$H-$^1$H COSY NMR spectrum of TCM-CPS in DMSO-$d_6$.

Figure S22 $^{13}$C NMR spectrum of TCM-CPS in DMSO-$d_6$. 
Figure S23 $^{19}$F NMR spectrum of TCM-CPS in DMSO-$d_6$.

Figure S24 HRMS spectrum of TCM-CPS.

Reference