Supporting Information

Pathological Biopsy Strategy by Regulating Intracellular ROS to Precisely Differentiate Cancer Cells from Diseased Tissues

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EXPERIMENTAL SECTION

General Information. Unless otherwise described, all reagents and solvents were purchased from commercial sources and used without further purification. All the samples were prepared according to the standard methods. Ultrapure water was used in all aqueous experiments. The molar concentration of LA was used to indicate the concentration of VC@cLAVs.

General Methods. The particle size and zeta potential were measured by a dynamic light scattering (DLS) analyzer (Malvern Zetasizer Nano ZS90, Britain). The content of the sulfur element in nanoparticles was obtained by ICP-OES (5100 SVDV, USA). The fluorescence images of intracellular ROS were observed using a fluorescent inverted microscope (Olympus IX73, Japan). The cell viability, relative ROS levels and chemiluminescence intensity were measured using a Thermo Varioskan Flash microplate reader (USA). The chemiluminescence images in HeLa, Raw 264.7, HUVEC cells were observed using a IVIS spectrum imaging system (PerkinElmer, USA). The statistical charts of luminescence intensity were analyzed by ImageJ software. The real-time chemiluminescence images of cells were observed using a confocal aser scanning microscopy (Zeiss LSM880, USA).

Reagents. Lipoic acid (LA, >99%), 1,4,7-triazacyclononane (>95%) and Vitamin C (VC, >99%) were purchased from Tansoole (Shanghai, China). Bis(2-carbopentyloxy-3,5,6-trichlorophenyl) oxalate (CPPO, 92%) and Pluronic F127 (Mn = 12600) were purchased from Aladdin (Shanghai, China). 2-(3,6-Diacetoxy-2,7-dichloro-9H-xanthen-9-yl)benzoic acid (DCFH-DA, 97%), 3-(4,5-Dimethyl-2-

Thiazolyl)-2,5-Diphenyl Tetrazolium Bromide (MTT, >99%) and Cyanine3 amine (Cy3, 95%) were purchased from Macklin (Shanghai, China). Roswell Park Memorial Institute (RPMI-1640), Dulbecco's modified eagle medium (DMEM), penicillin/streptomycin and pancreatin were purchased from Gibco (USA). The FBS and phosphate buffered saline (PBS) were purchased from Biological Industries (Israel). Hoechst 33342 was purchased from Beyotime Biotechnology.

Typical Preparation of VC@cLAVs. The synthesis details, characterization data and drug loading capacity of VC@cLAVs were mentioned in previous work.[1]

Typical Preparation of CPPO@F127. The synthesis of CPPO@F127 was following previous report with minor modifications.[2] Pluronic F-127 (10 mg), bis(2-carbopentyloxy-3,5,6-trichlorophenyl) oxalate (CPPO, 1 mg) and Cyanine3 amine (Cy3, 0.02 mg) were fully dissolved in THF (1 mL) and then the reaction mixtures were stirred for 2 h at room temperature. Finally, the white solid was concentrated by rotary evaporation, and then was mixed with phosphate buffered saline (PBS) to afford an aqueous dispersion of self-assembled CPPO@F127.

Dilution Stability of VC@cLAVs. The dilution stability of VC@cLAVs was evaluated by diluting the concentration below its critical aggregation concentration (200 μM). Briefly, the VC@cLAVs ([LA] = 2000 μM) was diluted to the concentrations of 2,000, 1,000, 500, 250, 125, 63, 32, 16, 8, 4, 2 and 1 μM, respectively. Afterward, the particle sizes of above solutions were recorded by DLS to evaluate its stability.

Dilution Stability of CPPO@F127. The CPPO@F127 ([CPPO] = 2000 μM) was

diluted to the concentrations of 2,000, 1,000, 500, 250, 125, 63, 32, 16, 8, 4, 2 and 1 μM, respectively. Afterward, the particle sizes of above solutions were recorded by DLS to evaluate its stability.

***In vitro* Cytotoxicity Assay.** The cell viability was detected by MTT assay. HeLa, HT29, A549, GL261, MCF-7, HepG2, HUVEC, L02, 16HBE and Raw 264.7 cells were seeded in 96-well plates (5×10^3 cells/well) and incubated at 37 ˚C/5 % CO_2 for 24 h, respectively. Next, the cells were treated with fresh media containing different concentrations of VC@cLAVs or CPPO@F127 for 12 h. After treatment, culture media was removed, then fresh media containing MTT solution (5 mg/mL) was added to each well and the plates were incubated for another 4 h. After incubation, the culture media were removed and crystals were then dissolved in 150 μL DMSO/well. The absorbance of the solution was measured at 490 nm using a Thermo Varioskan Flash microplate reader. Cell viability was calculated according to the following formula: cell viability (%) = A_{490} (sample)/A_{490} (control) × 100%.

Cellular Uptake behaviors of CPPO@F127.

The cellular uptake behaviors of the CPPO@F127 (Cy3, λ_{ex} = 550 nm, λ_{em} = 570 nm) were determined by confocal laser scanning microscopy (CLSM). The HeLa cells (3×10^4 cells/well) were seeded in Φ = 35 mm a glass Petri dish and cultured at 37 ˚C/5 % CO_2 for 12 h (n = 5). The old media were replaced with 1 mL of serum-free media containing CPPO@F127 (500 μM) for 0.5, 1, 2, 4 and 6 h. After, the fluorescence images were obtained using a CLSM.

Measurement of Intracellular Reactive Oxygen Species (ROS) by DCFH-DA.

The ROS levels in cells after incubation with VC@cLAVs at different times were investigated using 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA, λ_{ex} = 488 nm, λ_{em} = 525 nm).

The related intracellular ROS levels: HeLa, HT29, A549, GL261, MCF-7, HepG2, HUVEC, L02, 16HBE and Raw 264.7 cells were seeded in 96-well plates (5×10^3 cells/well) and incubated at 37 ˚C/5 % CO_2 for 24 h, respectively. Raw 264.7 cells were treated with lipopolysaccharide (LPS, 1 μg/mL) in serum-free medium for 12 h to get oxidative stress model. Next, the cells were treated with fresh media containing VC@cLAVs (500 μM) for 6 h. After, the cells were stained with DCFH-DA for 30 min at different times. The fluorescence intensity were obtained using a thermo varioskan flash microplate reader. The fluorescence intensity of DCFH-DA in cells at 0 min was normalized to 100% for each group.

The fluorescence images of intracellular ROS: HeLa, Raw 264.7 and HUVEC cells were seeded in 6-well plates (5×10^4 cells/well) and cultured at 37 ˚C/5 % CO_2 for 24 h. Raw 264.7 cells were treated with lipopolysaccharide (LPS, 1 μg/mL) in serum-free medium for 12 h to get oxidative stress model. The old media were replaced with 2 mL of serum-free media containing VC@cLAVs (500 μM) for 6 h. After, the cells were stained with DCFH-DA for 30 min at different times. After washing with PBS, the cell images were obtained on a fluorescent inverted microscope. The fluorescence images of DCFH-DA in HeLa cells, LPS-induced Raw 264.7 cells and HUVEC cells without any treatment was set as the control group.

Measurement of Intracellular ROS by Chemiluminescence. The real-time ROS

levels in cells after incubation with VC@cLAVs at different time were investigated using CPPO@F127 by chemiluminescence.

The chemiluminescence intensity of intracellular ROS: HeLa, HT29, A549, GL261, MCF-7, HepG2, HUVEC, L02, 16HBE and Raw 264.7 cells were seeded in 96-well plates (5×10^3 cells/well) and incubated at 37 °C/5 % CO_2 for 24 h, respectively. Raw 264.7 cells were treated with lipopolysaccharide (LPS, 1 μg/mL) in serum-free medium for 12 h to get oxidative stress model. Next, the cells were treated with fresh media containing VC@cLAVs (500 μM) for 6 h. Then, the cells were stained CPPO@F127 for 2 h. The chemiluminescence intensity was obtained at 20, 40, 60, 80, 100, 120 and 140 minutes using a Thermo Varioskan Flash microplate reader.

The chemiluminescence images of intracellular ROS levels: HeLa, Raw 264.7 and HUVEC cells were seeded in 48-well plates (1×10^4 cells/well) and cultured at 37 °C/5 % CO_2 for 24 h. Raw 264.7 cells were treated with lipopolysaccharide (LPS, 1 μg mL-1) in serum-free medium for 12 h to get oxidative stress model. The old media were replaced with serum-free media containing VC@cLAVs (500 μM) for 6 h. After incubation, the cells were stained CPPO@F127 for 2 h. The chemiluminescence images in HeLa, Raw 264.7 and HUVEC cells were observed using a IVIS spectrum imaging system.

Typical Procedure for the Real-Time Chemiluminescence Images of Cells. All cells (3×10^4 cells/well) were seeded in a glass Petri dish (Φ = 35 mm) and cultured at 37 °C/5 % CO_2 for 24 h. The old media were replaced with 1 mL of serum-free media containing H_2O_2 (100 μM) for 0.5 h or VC@cLAVs (500 μM) for 6 h. After

incubation, the cells were stained CPPO@F127 for 2 h. Finally, the cells were incubated with Hoechst 33342 solution prepared with fresh serum-free medium for 30 min. The chemiluminescence images were obtained for 160 or 120 minutes using a confocal laser scanning microscopy. Note: For the chemiluminescence images of mixed cells, GFP-HeLa and HUVEC cells mixed cells (3×10^4 cells/well, $n_{HeLa}/n_{HUVEC} = 1 : 2$) were seeded in a glass Petri dish.

Typical Procedure Incubation of Primary Cells from Tumor Tissue:

a. 10 mm^3 fresh tumor tissue was immersed in a culture dish. And necrotic tissue, calcified tissue and blood clots were removed with scissors. Remaining tissue repeatedly rinsed with D-Hanks solution containing penicillin and streptomycin.

b. The specimens were cut into small pieces $\leq$ 1 mm^3 with surgical scissors, repeatedly rinsed with D-Hanks solution and ground using the grinding rod so that the cells were fully filtered through a 200-mesh stainless steel cell sieve.

c. Then, tissue blocks were placed in centrifuge tubes containing 5 ml of trypsin and subsequently placed in a constant temperature shaker at 37 ˚C to digest the tissue blocks with different times for different tumor tissues. When the tissue block was well permeable and flocculent, it was centrifuged for 5 min with 1500 rpm/min. The supernatant was discarded and the cells were collected.

d. The collected cells were incubated with erythrocyte lysate for 1 min, followed by the addition of D-Hanks solution and centrifugation to remove erythrocytes, some dead cells and tissue debris. The cells were collected.

e. The collected cells were incubated with DNase and RNase for a few minutes,

and then it was centrifuged for 5 min with 1500 rpm/min. The supernatant was discarded and the cells were collected.

f. The collected cells were added DMEM complete medium to resuspend the cells and culture 10^5 cells in a glass bottom culture dish (Dish diameter = 35 mm, Glass diameter = 15 mm) for 6 h.

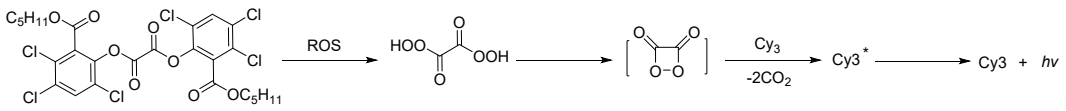
g. The old media were replaced with 0.5 mL of serum-free media containing VC@cLAVs (500 μM) for 6 h. Then, the cells were stained CPPO@F127 for 2 h. Finally, the cells were incubated with Hoechst 33342 solution prepared with fresh serum-free medium for 30 min. The chemiluminescence images of the corresponding cells were observed using a confocal laser scanning microscopy.

Statistical Analysis. Results were presented as the mean ± standard deviation (SD) of at least three trials. One-way analysis of variance (ANOVA) was used for statistical analysis with $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ and $****p < 0.0001$. And $p > 0.05$ represent no statistical significance.

References

1. C. Liao, X. Wang, X. Zhou, D. Wang, Z. Zhang, Y. Liu, X. Wu, Y. Chen, Y. Tan, X. Dai, P. Jing, J. Pang, X. Xiao, J. Liu, X. Liao, S. Zhang, *ACS Appl. Mater. Interfaces,* **2022**, 14, 49508-49520.

2. C. Lim, Y. Lee, J. Na, J. Oh, S. Her, K. Kim, K. Choi, S. Kim,* I. Kwon, *Adv. Funct. Mater.*, **2010**, 20, 2644-2648.



Scheme S1. The possible mechanism diagram of ROS-induced CPPO@F127 chemiluminescence.

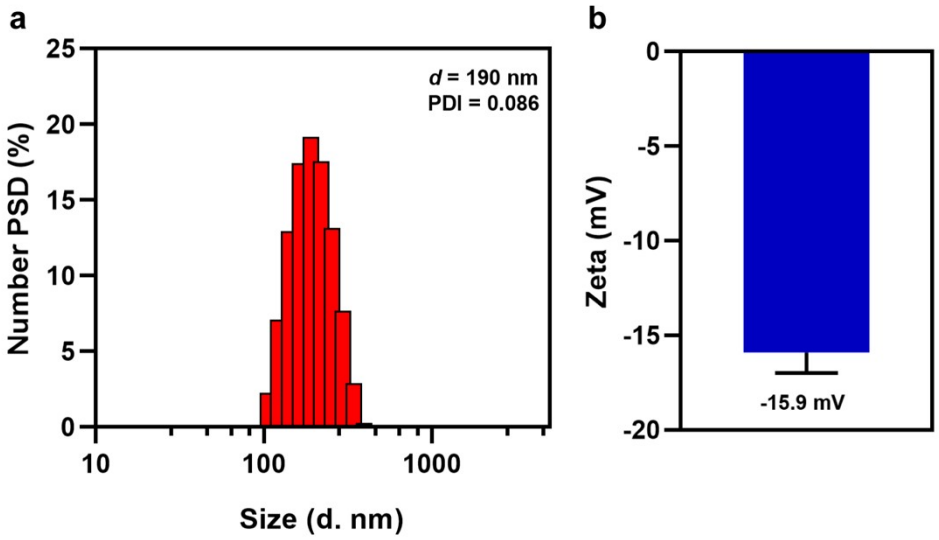


Fig. S1 (a) Distribution of hydrodynamic diameters of VC@cLAVs. (b) Zeta potential of VC@cLAVs.

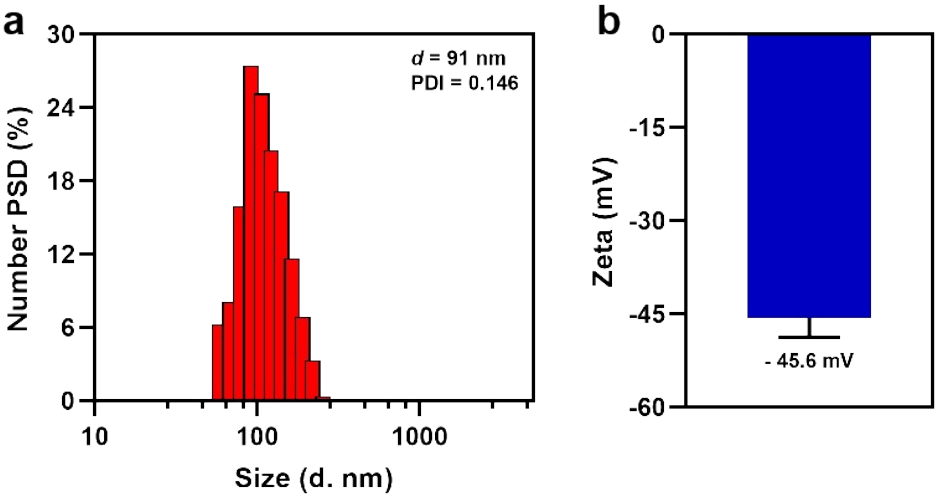


Fig. S2 (a) Distribution of hydrodynamic diameters of CPPO@F127. (b) Zeta potential of CPPO@F127.

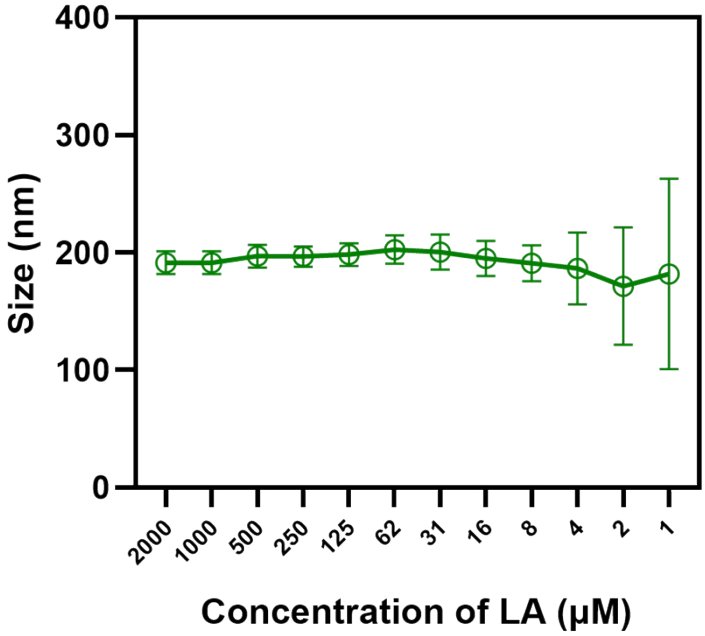


Fig. S3 Size of VC@cLAVs as a function of dilution factor.

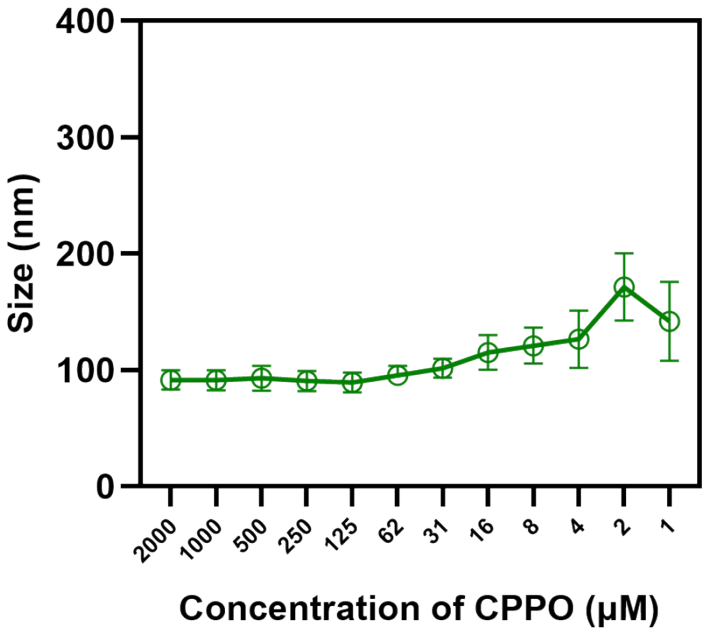


Fig. S4 Size of CPPO@F127 as a function of dilution factor.

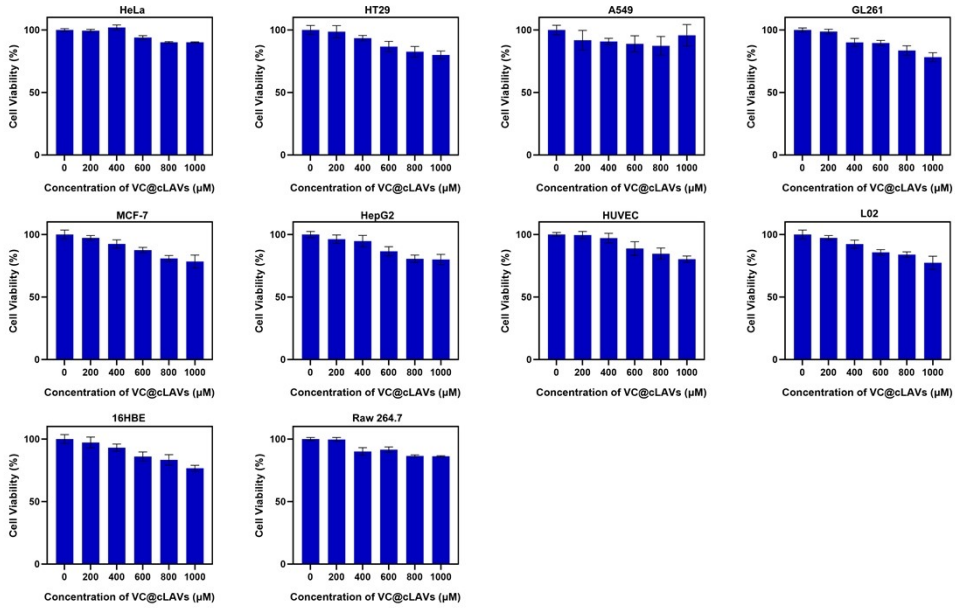


Fig. S5 Cell viability of HeLa, HT29, A549, GL261, MCF-7, HepG2, HUVEC, L02, 16HBE and Raw 264.7 cells after incubation with VC@cLAVs for 12 h detected by MTT assay (mean ± SD, n = 6).

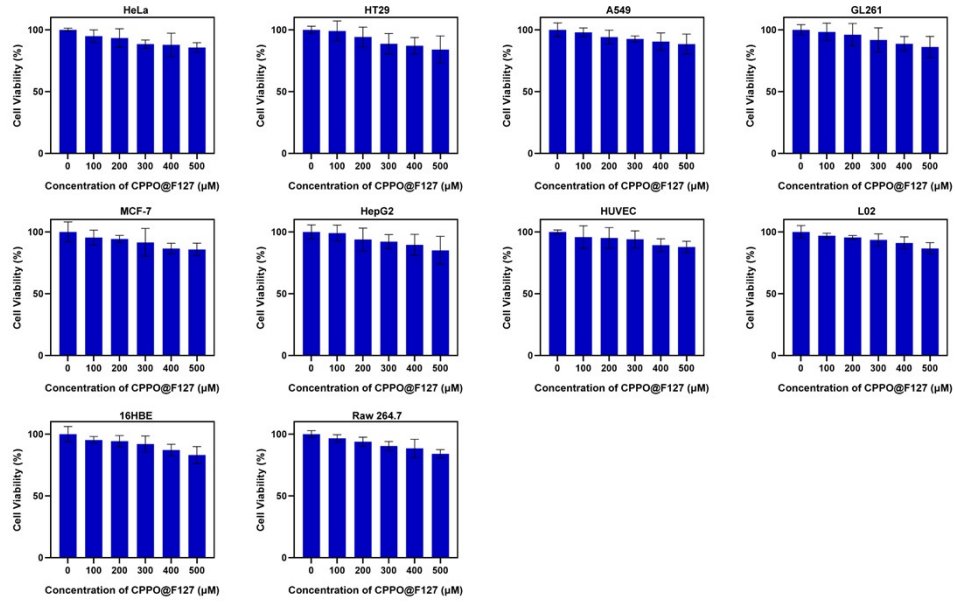


Fig. S6 Cell viability of HeLa, HT29, A549, GL261, MCF-7, HepG2, HUVEC, L02, 16HBE and Raw 264.7 cells after incubation with CPPO@F127 for 12 h detected by MTT assay (mean ± SD, n = 6).

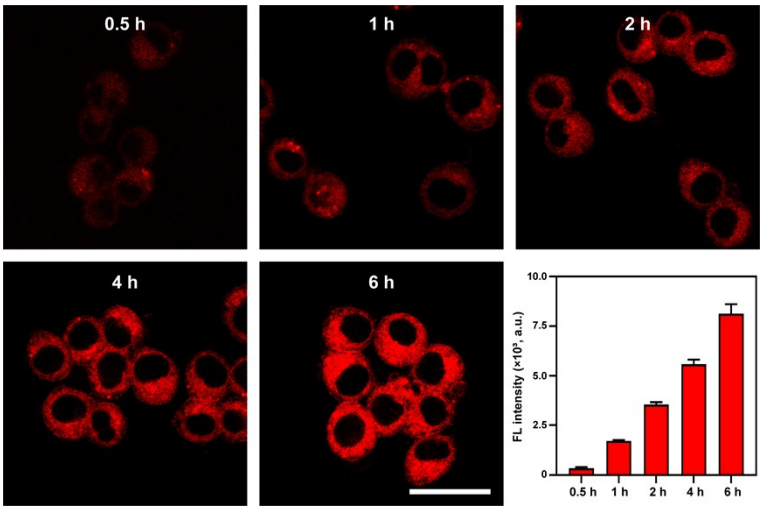


Fig. S7 Fluorescent images and quantified fluorescence intensity of HeLa cells treating with CPPO@F127 for 0.5, 1, 2, 4 and 6 h (n = 5). Scale bar: 50 μm.

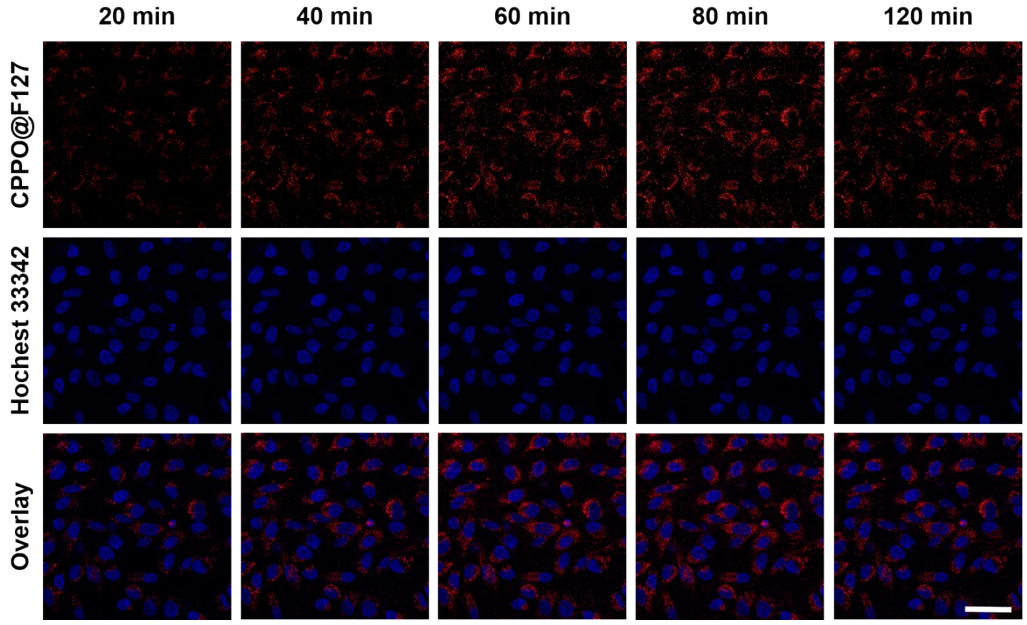


Fig. S8 The real-time chemiluminescence images of HeLa cells in the same field of view within 120 min after treatment with VC@cLAVs for 6 h followed by incubation with CPPO@F127 using CLSM. Scale bar: 50 μm.

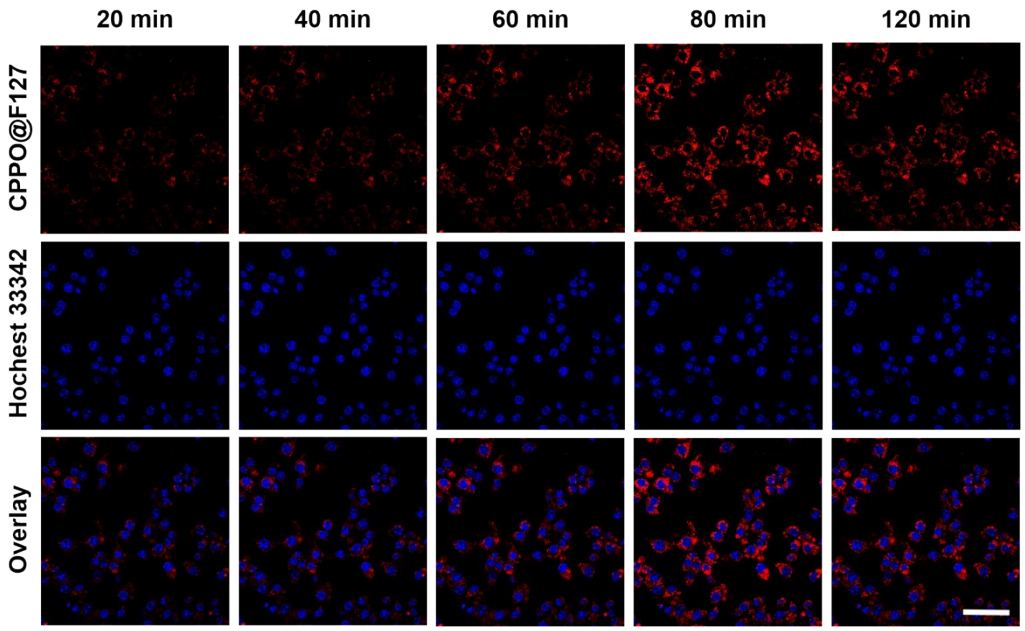


Fig. S9 The real-time chemiluminescence images of HT29 cells in the same field of view within 120 min after treatment with VC@cLAVs for 6 h followed by incubation with CPPO@F127 using CLSM. Scale bar: 50 μm.

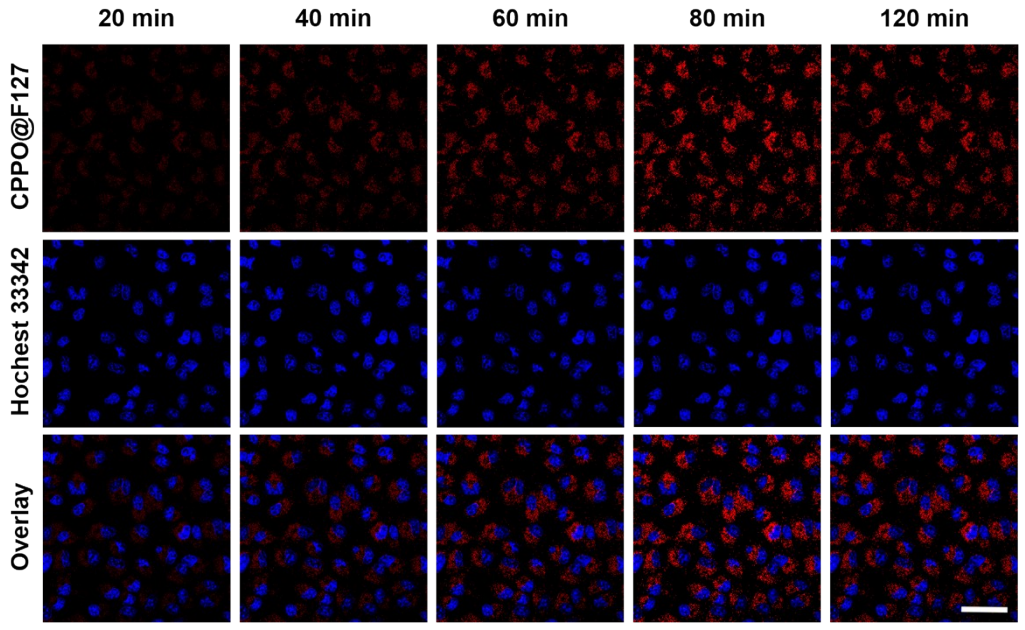


Fig. S10 The real-time chemiluminescence images of A549 cells in the same field of view within 120 min after treatment with VC@cLAVs for 6 h followed by incubation with CPPO@F127 using CLSM. Scale bar: 50 μm.

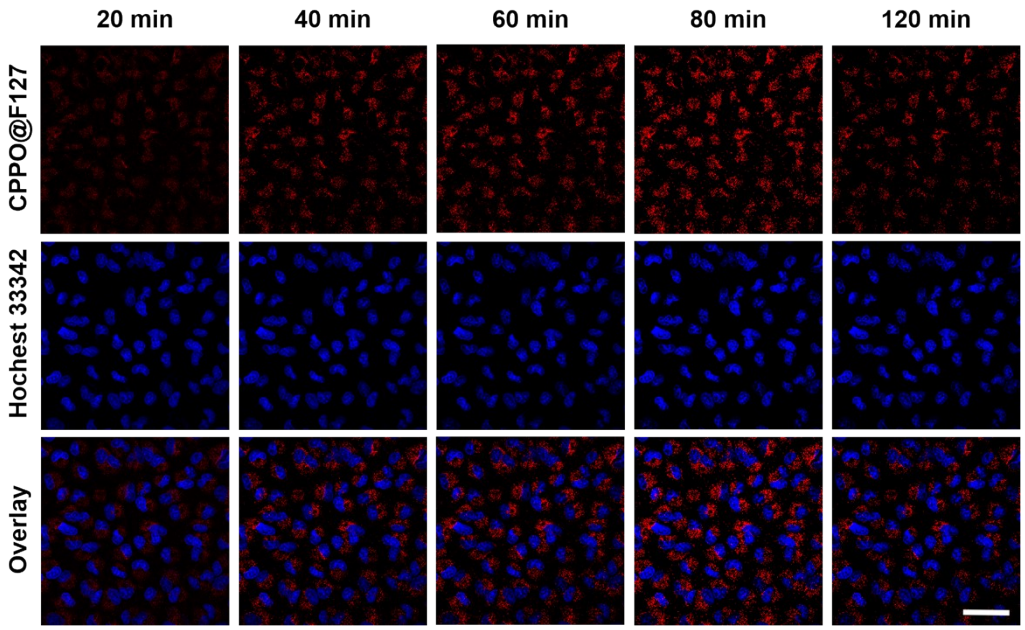


Fig. S11 The real-time chemiluminescence images of GL261 cells in the same field of view within 120 min after treatment with VC@cLAVs for 6 h followed by incubation with CPPO@F127 using CLSM. Scale bar: 50 μm.

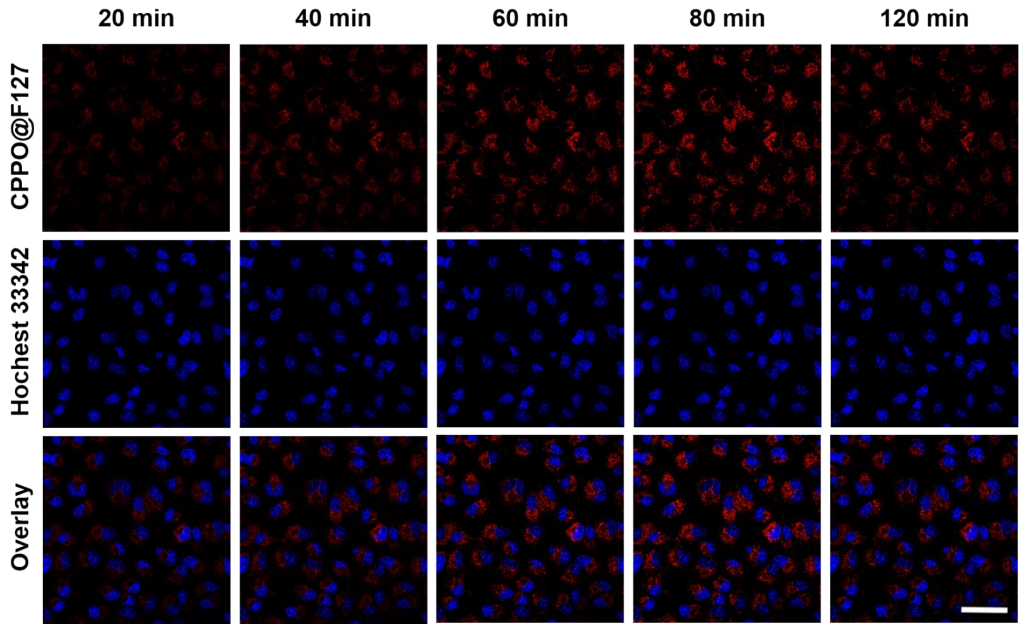


Fig. S12 The real-time chemiluminescence images of MCF-7 cells in the same field of view within 120 min after treatment with VC@cLAVs for 6 h followed by incubation with CPPO@F127 using CLSM. Scale bar: 50 μm.

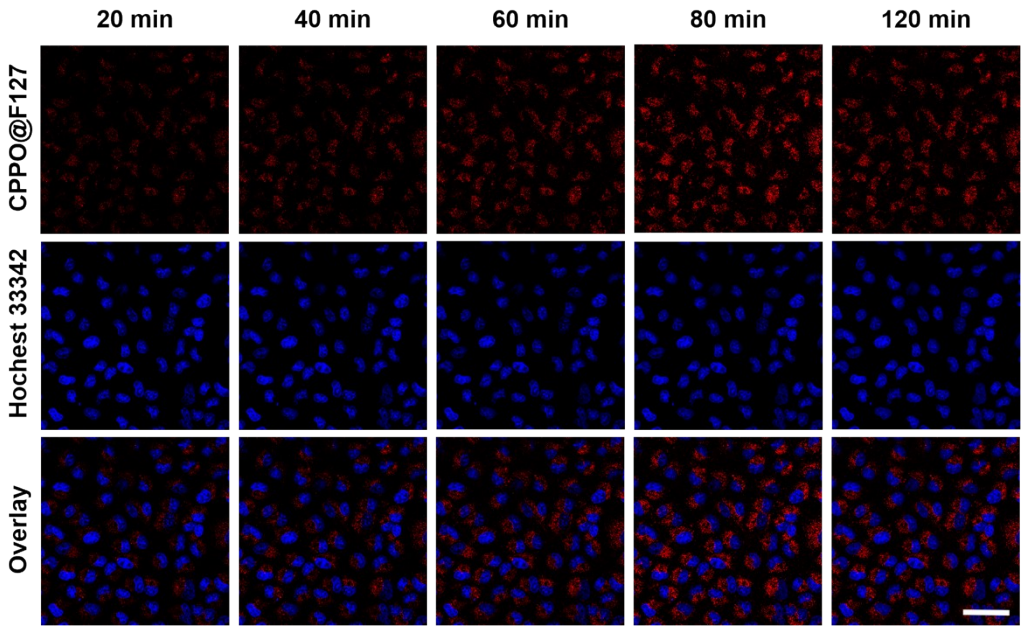


Fig. S13 The real-time chemiluminescence images of HepG2 cells in the same field of view within 120 min after treatment with VC@cLAVs for 6 h followed by incubation with CPPO@F127 using CLSM. Scale bar: 50 μm.

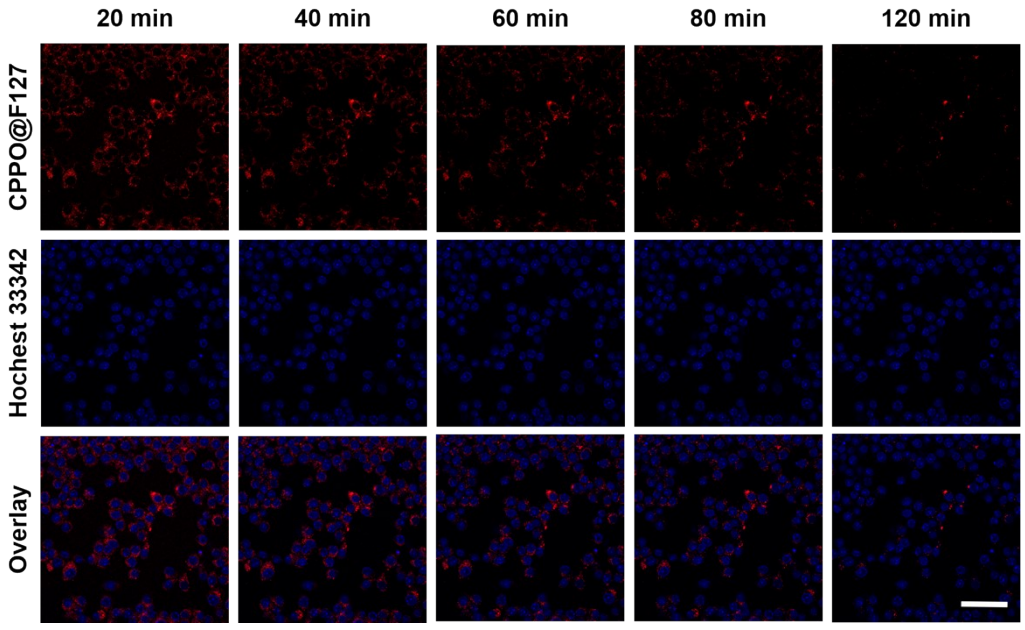


Fig. S14 The real-time chemiluminescence images of Raw 264.7 cells in the same field of view within 120 min after treatment with VC@cLAVs for 6 h followed by incubation with CPPO@F127 using CLSM. Scale bar: 50 μm.

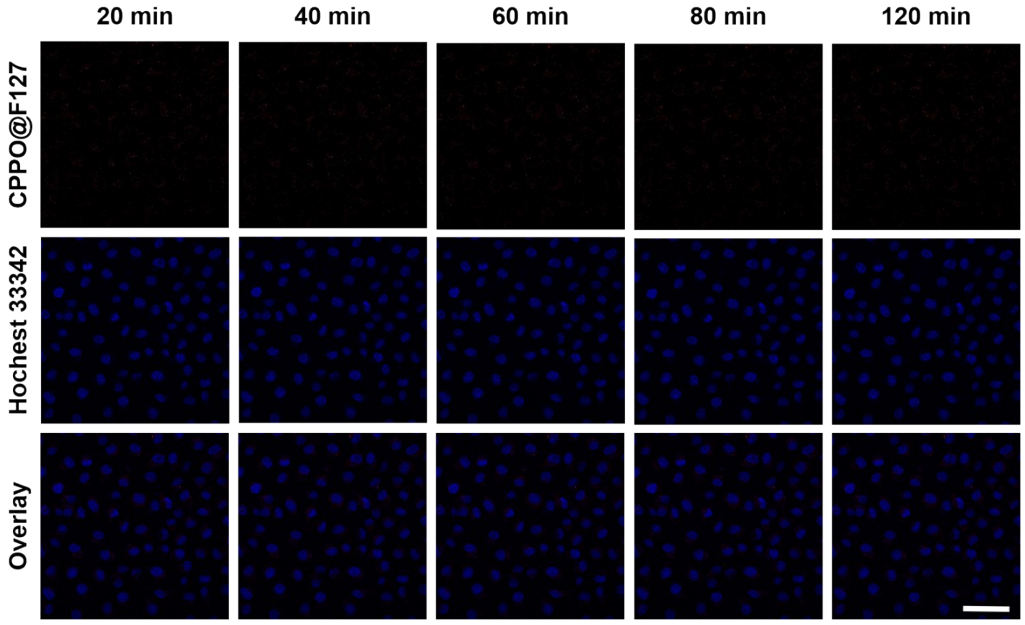


Fig. S15 The real-time chemiluminescence images of HUVEC cells in the same field of view within 120 min after treatment with VC@cLAVs for 6 h followed by incubation with CPPO@F127 using CLSM. Scale bar: 50 μm.

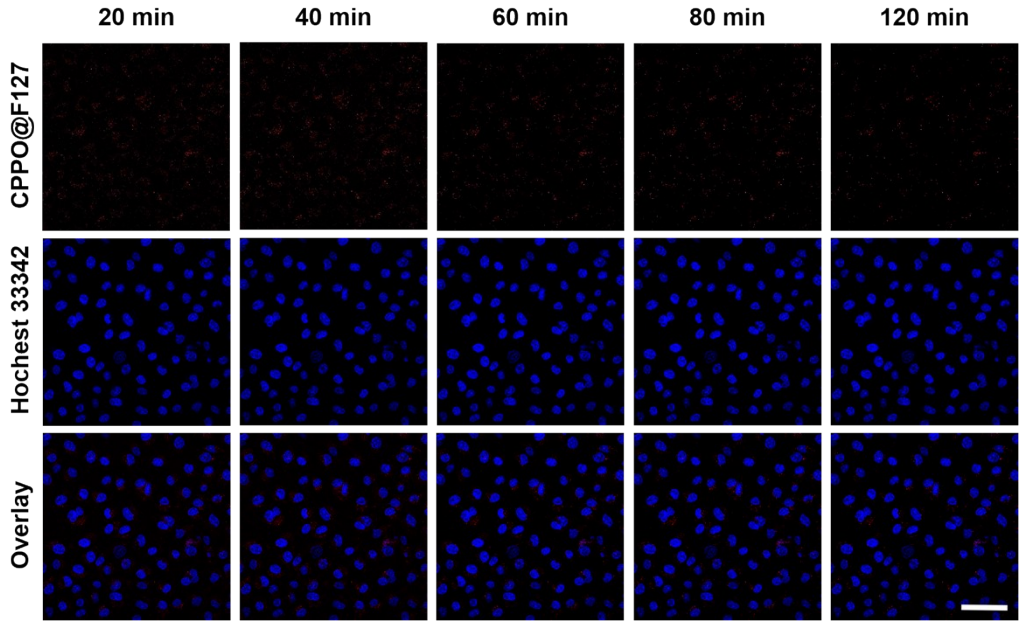


Fig. S16 The real-time chemiluminescence images of L02 cells in the same field of view within 120 min after treatment with VC@cLAVs for 6 h followed by incubation with CPPO@F127 using CLSM. Scale bar: 50 μm.

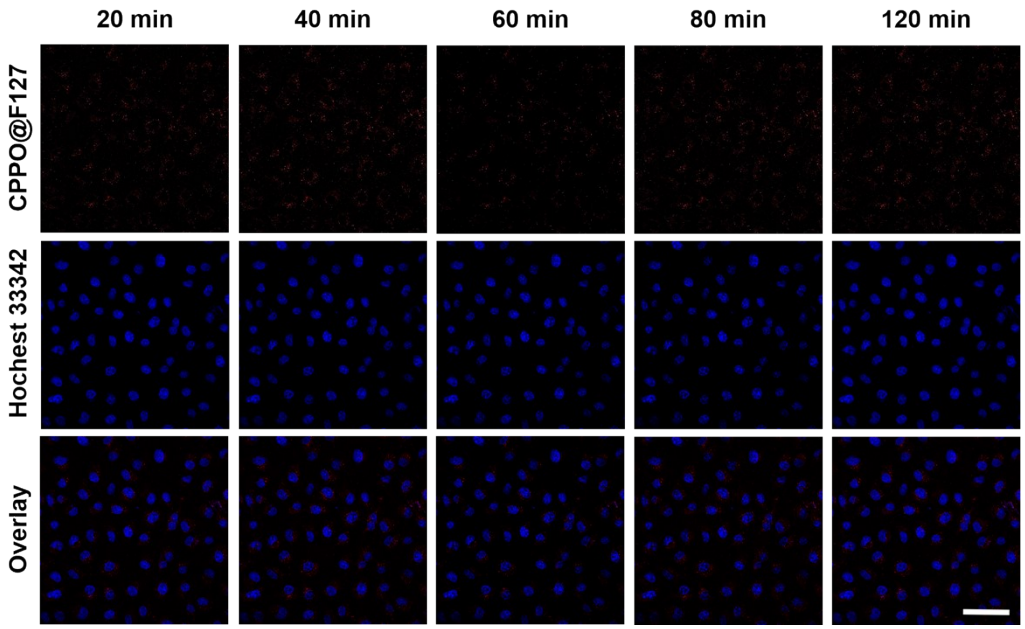


Fig. S17 The real-time chemiluminescence images of 16HBE cells in the same field of view within 120 min after treatment with VC@cLAVs for 6 h followed by incubation with CPPO@F127 using CLSM. Scale bar: 50 μm.

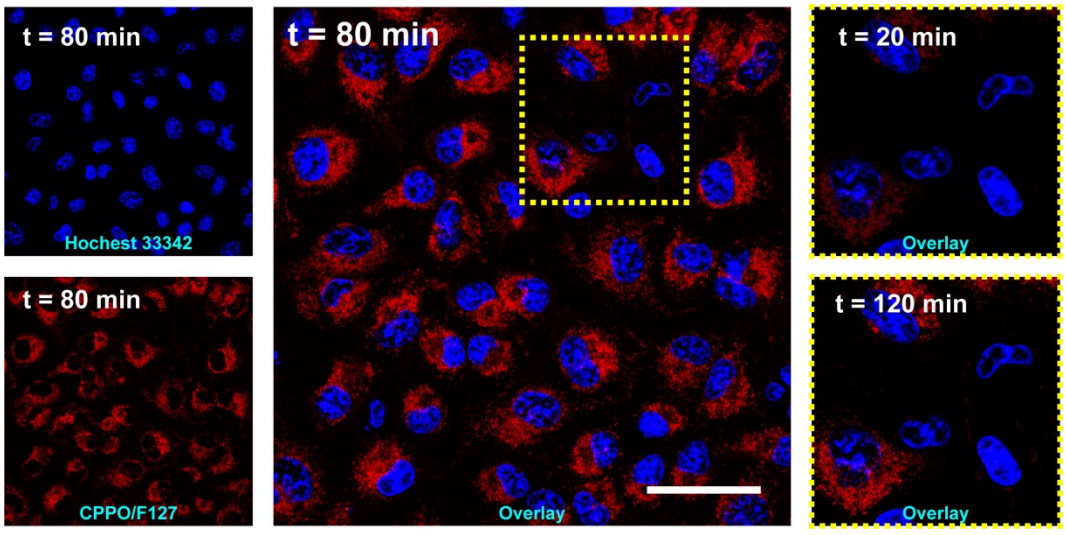


Fig. S18 The chemiluminescence images of A549 cells extracted from tumor-bearing mice in the same field of view at 20 min, 80 min and 120min after treatment with VC@cLAVs for 6 h followed by incubation with CPPO@F127 using CLSM. The right virtual frames of each image represent the focus of the area shown by the yellow

virtual frame in the image. Scale bar: 50 μm.

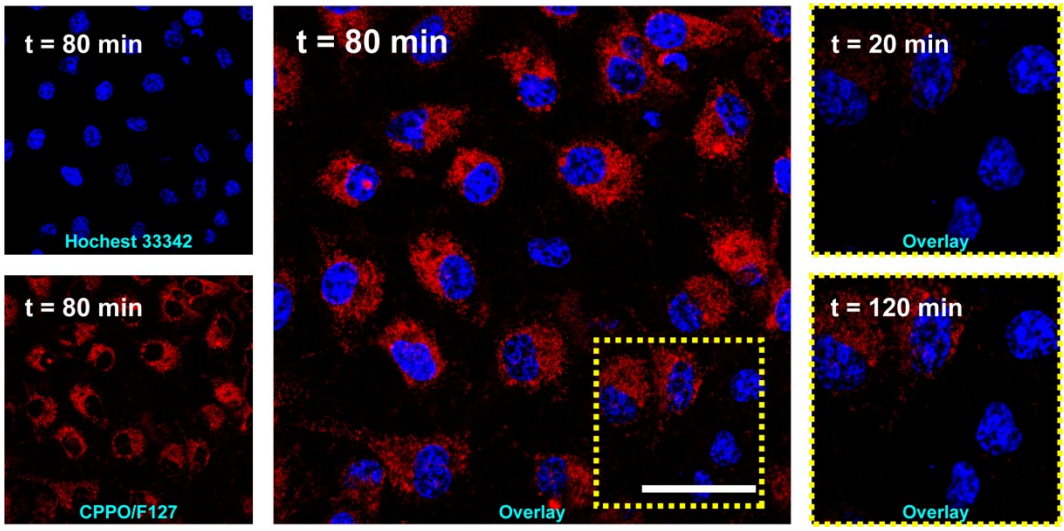


Fig. S19 The chemiluminescence images of HepG2 cells extracted from tumor-bearing mice in the same field of view at 20 min, 80 min and 120 min after treatment with VC@cLAVs for 6 h followed by incubation with CPPO@F127 using CLSM. The right virtual frames of each image represent the focus of the area shown by the yellow virtual frame in the image. Scale bar: 50 μm.

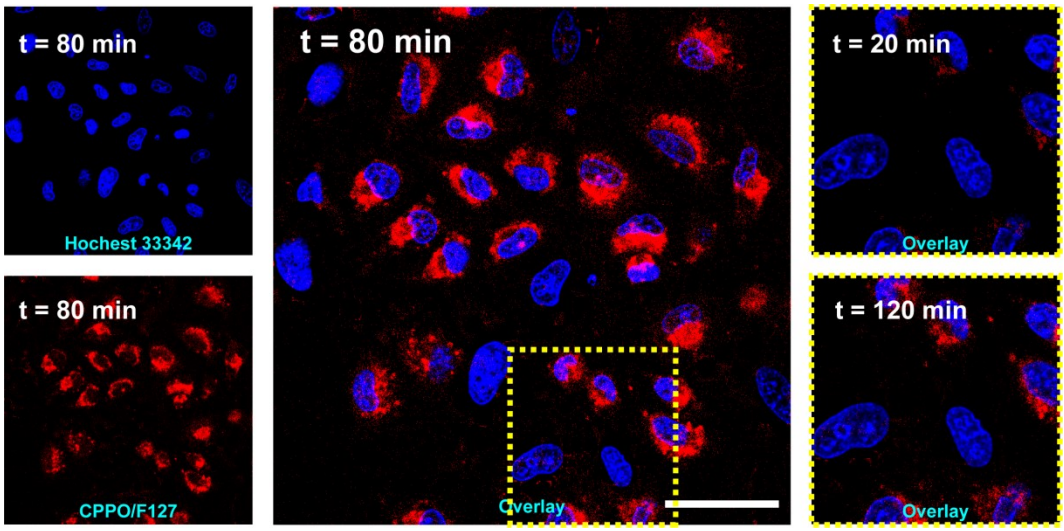


Fig. S20 The chemiluminescence images of HT29 cells extracted from tumor-bearing mice in the same field of view at 20 min, 80 min and 120 min after treatment with

VC@cLAVs for 6 h followed by incubation with CPPO@F127 using CLSM. The right virtual frames of each image represent the focus of the area shown by the yellow virtual frame in the image. Scale bar: 50 μm.

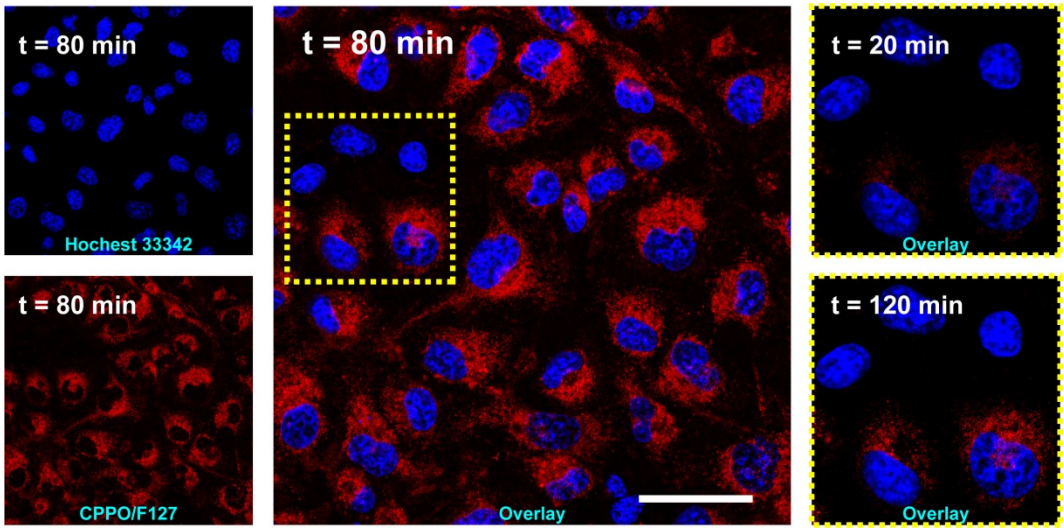


Fig. S21 The chemiluminescence images of MCF-7 cells extracted from tumor-bearing mice in the same field of view at 20 min, 80 min and 120 min after treatment with VC@cLAVs for 6 h followed by incubation with CPPO@F127 using CLSM. The right virtual frames of each image represent the focus of the area shown by the yellow virtual frame in the image. Scale bar: 50 μm.