Water-Dispersible Fluorescent COFs Disturb Lysosomal Autophagy to Boost Cascading Enzymatic Chemodynamic-Starvation Therapy

Hui Liu[a], Wenxin Lv[a], Darambazar Gantulga[b], Yi Wang*[a]

[a] Center for Advanced Low-dimension Materials, State Key Laboratory for Modification of Chemical Fibers and Polymer Materials, College of Chemistry and Chemical Engineering, Donghua University, Shanghai, 201600, China

[b] Department of Biology, School of Biomedicine, Mongolian National University of Medical Sciences, Zorig Street 2, Peace Avenue, Sukhbaatar district, Ulaanbaatar 14210, Mongolia

*Corresponding authors

Email: ywang@dhu.edu.cn

1. Materials and methods

1.1 Materials

3,5,3,5-tetramethylbenzidine (TMB), 1,3,5-tris(4-aminophenyl)benzene (TAPB) and 2,5-dimethoxyterephthalaldehyde (DMTP) were obtained from Leyan Regent Co., Ltd (China). 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride was purchased from TCI Co., Ltd (Japan) Anhydrous acetonitrile, N-Hydroxysuccinimide and acetic acid were obtained from Aladdin Regent Co., Ltd. Ethanol, Polyethyleneimine (PEI-600), potassium iodide, ammonium molybdate, ethanol absolute, sodium hydroxide, iron(III) chloride hexahydrate, sodium citrate dehydrate, isopropyl alcohol, and hydrogen peroxide were sourced from Shanghai Titan Science Co., Ltd. (China). Glucose oxidase (GOx), Cy5-SE, Cell Counting Kit-8, 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA), 4',6'-diamidino-2-phenylidole (DAPI), 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein,acetoxymethyl ester (BCECF-AM), and JC-1 mitochondrial potential sensor were acquired from MeiLunbio Co., Ltd. Aminophenyl fluorescein (APF) was purchased from MKbio Co., Ltd. Acridine Orange (AO) hydrochloride was obtained from MedChemExpress Co., Ltd. ATP assay kit (Colorimetric/Fluorometric) and Autophagy Staining Assay Kit were procured from Beyotimebio Co., Ltd. Cell culture medium 1640 and tyrisin were sourced from Gibco. PBS solution, Hank's, penicillin/streptomycin solution,

and L-glutamine were obtained from Hyclone. 4% sheep blood cells were acquired from Hongquan Biotechnology Co., Ltd.

1.2 Synthesis of Fe_2O_3

Firstly, 0.045 g $FeCl_3 \cdot {}_6H_2O$ and 0.1 g $Na_3C_6H_5O_7 \cdot 2H_2O$ were dissolved in 100 mL H_2O. Then, the pH of the solution was tuned to 5.8 with 0.1 M NaOH solution. After 0.024 g $CO(NH_2)_2$ was added which then continuously stirred and refluxed at 100℃ for 24 h. Then, the finished solution was then mixed with three times its volume of isopropanol for 5 h. The finished solution was centrifuged at 8000 rpm/min to isolate production after purification, which was then freeze-dried to obtain Fe_2O_3.

1.3 Synthesis of COF

Firstly, 3.55 mg DMTP and 3.4 mg TAPB were each dissolved in 950 μL acetonitrile. After ultrasonic dissolution, the two solutions were mixed together. With continuous stirring, a 10 mL solution of acetic acid [5% ($V_{acetic\ acid}/V_{water}$)] was dropped into the system. After dropwise addition, stop stirring and leave the system standing for 72 h at room temperature. Then, the precipitate was obtained at 11000 rpm/min and washed three times with alcohol and water. The resultant product was freeze-dried to obtain the COF. For the exploration of the optimal amount of acetic acid, the conditions mentioned above were kept constant, with only the concentration of acetic acid being varied [2.5%, 5%, 10%, 10%, 20%, ($V_{acetic\ acid}/V_{water}$)].

1.4 Synthesis of CF

Firstly, 10 mg COF and 10 mg PEI (Mw = 600) were respectively dispersed in 10 mL H_2O and stirred for 30 min. Then the finished system was centrifuged at 8000rpm/min and washed three times to obtain COF-PEI (CP). The 10 mg CP and Fe_2O_3 solution (1 mg/mL) were then re-dispersed and mixed in 20 mL deionized water and then sonicated for 10 min. Then, the precipitate was obtained at 8000 rpm/min and washed three times with alcohol and water which then was freeze-dried to obtain CF.

1.5 Synthesis of CG

Initially, 10 mg GOx, 6.3 mg EDC and 8.5 mg of NHS were mixed in 1 mL H_2O and stirred for 30 min at 37℃ to yield activated GOx. The activated GOx was mixed with CP solution and stirred at 37℃ for 8 h. Then, the finished solution was separated and precipitated, followed by three washes with water. The final product CG was obtained after freeze-drying.

1.6 Synthesis of CFG and CFGM

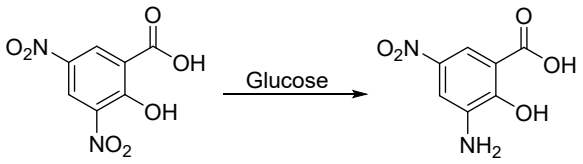
Firstly, 30 mg CF was dispersed in 60 mL deionized water with 10 mg activated GOx was mixing and stirring at 37℃ for 8 h. After the precipitate was washed three times with water and then freeze-dried to obtain CFG. To synthesize CFGM, 4T1 tumor cells were cultured in dishes until they reached confluence which then was digested with trypsin solution and washed thrice with PBS at 800 g for 5 min, and gathered. Then, subsequently dissolved in a low-osmotic lysis buffer containing Tris-HCl (20 mM), KCl (10 mM), $MgCl_2$ (2 mM), and a proteinase inhibitor, excluding EDTA. The cell suspension was sonicated in a cell disruptor for 15 min, sonicated at 10°C for 30 min, and the supernatant was obtained after 5 min of centrifugation which was then centrifuged for 30 min at 20000 g to yield purified cancer cell membrane (CM), where the protein concentrations were detected by BCA quantification test kit. The CM was re-suspended in 1x PBS and mixed with CFG which then filtered through a polycarbonate membrane with a 400 nm pore diameter. After centrifuging for 15 min at 11000 rpm, the precipitated CFGM was collected.

1.7 Characterizations

UV-vis absorption spectra were analyzed with Cary300 UV-vis Spectrophotometer from Agilent Technologies. The Zetasizer Nano-ZS90 from Malvern was used for determining the size distribution and zeta potential. X-ray photoelectron spectroscopy (XPS) data was obtained with the Thermal Fisher ESCALAB 250Xi. Transmission electron microscopy (TEM) and energy-dispersive spectroscopy (EDS) were performed on a FEI Talos F200S, while scanning electron microscopy (SEM) was conducted on a Hitachi Regulus 8230. For TEM and SEM imaging, the samples were diluted in ethanol and dropped onto a copper grid and clean tin foil, respectively. FTIR spectra were captured using the ATR attachment method on TensorFlow II. A Lambda 950 Spectrophotometer from PerkinElmer was used for measuring UV-vis absorption spectra. The Crystallinity of the materials was examined by X-ray diffraction (XRD) analysis on a Bruker D8 ADVANCE diffractometer using Cu Kα radiation.

1.8 Consumption of glucose

The glucose consumption of CG and CFG in solution was assessed using the 3,5-dinitrosalicylic acid (DNS) colorimetric method as follow. DNS will be reduced into 3-amino-5-nitrosalicylsaeure by glucose with the decreased adsorption at 540 nm. CG and CFG solutions of varying concentrations (0, 50, 100, 200 μg/mL) were added to 5 mM glucose solution for hydrolysis. At 0, 1, 3, 6, 12, and 24 h, 0.2 mL of each reaction system was mixed with 200 μL DNS. After heating at 100℃ for 5 min, the finished solution was diluted with 1 mL deionized water. Subsequently the absorbance of finished solution was determined at 540 nm.



DNS (540 nm↓)

1.9 H_2O_2 Detection

The generation of H_2O_2 was evaluated via iodometry, as H_2O_2 can oxidize I^- to I_3^- with increased adsorption at 352 nm in the presence of ammonium molybdate. KI solution (2 M, 1 mL) and ammonium molybdate solution (10 mM, 50 μL) were mixed to different concentrations CFG solution (0, 50, 100, 200 μg/mL; 5 mM glucose solution) and incubated for 30 min at 37℃. After centrifuging, the absorbance of the supernatant was determined at 352 nm.

$$H_2O_2 + 2I^- + 2H^+ \longrightarrow I_2 + 2H_2O$$

$$I_2 + I^- \overset{(NH)_4MoO_4}{\rightleftharpoons} I_3^- \ (352\ nm\uparrow)$$

1.10 pH decrease

Different concentrations of CFG solution (0, 50, 100, 200 μg/mL) were combined with various concentrations of glucose solution (0, 5, 10, and 50 mM). The pH of the reaction system was recorded at 0, 0.5, 1, 3, 5, and 8 h. The drop in pH indirectly demonstrated the glucose oxidase activity of the CFG.

$$O_2 + \text{Glucose} \xrightarrow{GOx} \text{Gluconic acid} + H_2O_2$$

(pH↓)

1.11 POD-like property detected by TMB

The generation of ·OH was assessed using 3,5,3,5-tetramethylbenzidine (TMB). Different concentrations of CF and CFG solutions (0, 50, 100, 200 μg/mL) were mixed with PBS (pH 5.0, pH 6.0, pH 7.4), TMB (0.5 mM), and H_2O_2 (10 mM) and then incubated for 30 min at 37℃. In a similar manner, CFG (200 μg/mL) solution was reacted with 5 mM glucose solution and TMB (0.5 mM) for 30 min at 37℃. After centrifuging, the absorbance of the supernatant was determined at 652 nm.

1.12 CAT-like property

The alterations in dissolved oxygen were measured in CF solution (pH 7.4) containing H_2O_2 (10 mM), CG solution containing glucose (5 mM), and CFG containing glucose (5 mM), respectively. These measurements were taken at 37℃ using a dissolved oxygen meter.

1.13 Cell culture and animals

4T1 tumor cells were acquired from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences. The cells were cultivated in a 5% CO_2 environment at 37°C in RPMI-1640 medium containing 1% penicillin/streptomycin and 10% fetal bovine serum. 20-g Female Balb/c mice aged 6-8 weeks were maintained under specific pathogen-free conditions within the Department of Laboratory Animals. **All animal experiments complied with the guidelines evaluated and sanctioned by the Ethics Committee of Donghua University (Number: DHUEC-NSFC-2021-16).**

1.14 Cellular uptake and localization

4T1 cells (1×10^5 cells/well) were seeded into confocal dishes containing 400 μL of culture medium and cultured overnight. Cell medium containing CFG and CFGM (500 μL, 20 μg/mL) was added and incubated for 2 and 4 h using a countdown method. After incubating, the cells were then treated with Hoechst33258 (200 μL, 10 μM) for 15 min, and subsequently incubated for 20 min with LysoTracker Red DND-99 (200 μL, 50 nM). After washing with PBS, Carl Zeiss LSM710 instrument was used for imaging the cells. The green channel, representing CFGM, was excited by 405 nm light and emitted at 515 nm. The red channel, representing LysoTracker Red DND-99, was excited by 561 nm light and emitted at 597 nm. The blue channel, representing Hoechst33258, was excited by 352 nm light and emitted at 460 nm. Intracellular fluorescence values and lysosomal fluorescence values for CFG and CFGM were determined by flow cytometry.

1.15 In vitro cytotoxicity assay

To assess the cytotoxicity of each material, CCK-8 kit was acquired to determine cell viability. 4T1 cells (1×10^4 cells/well) were seeded into a 96-well plate containing 200 μL of culture medium and cultured for 24 h. With spent medium discarded, the cell medium containing COF, CF, CG, CFG, and CFGM (0, 10, 20, 40, 80, 100 μg/mL respectively) was added and incubated 4 h. After replacing with fresh cell medium, the cells were cultured for an additional 20 h. After discarding the spent medium, CCK-8 solution (100 μL, in a 1:10 ratio with the medium) was added and incubated for 1 h. BioTek instrument was used for determining the absorbance at 450 nm.

1.16 Live-dead cells visualization

Cell viability was determined by live/dead cell staining kit (Calcein-AM, PI). 4T1 cells (1×10^4 cells/well) were seeded into 96-well plate which containing 200 μL of culture medium and cultured for 24 h. The spent medium was discarded, and the cell culture medium containing CFGM was added. After replacing with fresh cell medium, the

cells was cultured for an additional 20 h. Calcein-AM (2 μM, 100 μL) and PI (4 μM, 100 μL), both diluted in PBS, were added and incubated for 20 min, followed by two washes. Live cells (Calcein-AM at 515 nm, green) and dead cells (PI at 617 nm, red) were imaged with Carl Zeiss LSM710 instrument at 495 nm and 535 nm respectively. 4T1 cells that received no treatment served as the control group.

1.17 Intracellular ·OH, total ROS, H_2O_2 and pH detection

4T1 cells (1×10^6 cells/well) were seeded into 6-well plate which containing 2 mL of culture medium and cultured for 24 h. The spent medium was discarded and replaced with COF, CF, CG, CFG, and CFGM (500 μL, 20 μg/mL) for a 4-h incubation period. Afterwards, the cells were then incubated with various probes, including DCFH-DA (total ROS probe), APF (•OH probe), ROS Green™ (H_2O_2 probe), and BCECF-AM (pH probe) staining 30 min to 1 h. Finally, the cells were imaged with Carl Zeiss LSM710 instrument (DCFH-DA: Excitation at 488 nm, emission at 525 nm; APF: Excitation at 490 nm, emission at 515 nm; ROS Green™: Excitation at 490 nm, emission at 525 nm; BCECF-AM: Excitation 488 nm, emission 535 nm).

1.18 Measurement of mitochondrial membrane potential

JC-1 fluorescence probe was used for assessing mitochondrial membrane potential (MMP) changes. 4T1 cells (1×10^5 cells/well) were seeded into confocal dishes containing 400 μL of culture medium and cultured for 24 h. After replacing with cell culture medium containing COF, CF, CG, CFG, or CFGM (500 μL , 20 μg/mL), the cells were incubated for an additional 4 h. Then, after treating with JC-1 probe (200 μL, 20 μM), diluted in JC-1 and washing twice, the cells were imaged with Carl Zeiss LSM710 instrument. The red fluorescence signal images of JC-1 aggregates were captured at 590 nm under 585 nm light excitation, and the green fluorescence signal images of JC-1 monomers were captured at 529 nm under 514 nm light excitation. 4T1 cells that received no treatment served as the control group.

1.19 Detection of lysosomal membrane permeabilization

Lysosomal membrane permeability (LMP) was assessed using Acridine Orange (AO). 4T1 cells (1×10^5 cells/well) were seeded into confocal dishes containing 400 μL of culture medium and cultured for 24 h. With Cell culture medium containing COF, CF, CG, CFG, or CFGM (20 μg/mL, 500 μL) replacing, the cells were incubated for an additional 4 h. Subsequently, the cells were treated with AO probe (200 μL, 7.5 μg/mL) diluted in PBS and then incubated for 20 min. Then, the cells were imaged with Carl Zeiss LSM710 instrument after washing twice with PBS. The green fluorescence signals of dsDNA collected at 530 nm and orange fluorescence signals of ssDNA and RNA collected at 640 nm, both under 488 nm light excitation. 4T1 cells that received no treatment served as the

control group.

1.20 Detection of autophagy

The level of autophagy was assessed using the autophagy fluorescence probe, Monodansylcadaverine (MDC). 4T1 cells (1×10^5 cells/well) were seeded into confocal dishes containing 400 μL of culture medium and cultured for 24 h. With cell culture medium containing COF, CF, CG, CFG, and CFGM (500 μL, 20 μg/mL) replacing, the cells incubated for an additional 4 h. Subsequently, MDC probe (200 μL, 1×) diluted in Assay Buffer (1×) was added and the cells were incubated for 20 min, repeated twice. The cells were imaged with Carl Zeiss LSM710 instrument (Ex 330 nm, Em 510 nm) after washing twice with cell culture medium,. 4T1 cells that received no treatment served as the control group. After subjecting to the same steps repeatedly, the protein lysate was collected for Western blotting to analyze the expression of LC3B in 4T1 cells treated with different nanomaterials.

4T1 cells (1×10^5 cells/well) were seeded into confocal dishes containing 400 μL of culture medium and cultured for 24 h. With COF-containing cell culture medium (500 μL, 20 μg/mL) at different pH (7.4, 6.5, and 5.0) replacing, the cells were incubated for an additional 4 h. With MDC probe diluted in Assay Buffer adding, the cells were incubated for 20 min. The cells were imaged with Carl Zeiss LSM710 instrument (Ex 335 nm, Em 512 nm) after washing twice with cell culture medium. 4T1 cells treated at different pH (7.4, 6.5, and 5.0) served as the control group.

1.21 Evaluation of ATP production

Intracellular ATP content was determined via ATP detection kit. 4T1 cells were seeded into 100 mm petri dishes containing 200 μL culture medium and cultured for 24 h. With cell culture medium containing COF, CF, CG, CFG, or CFGM (100 μL, 20 μg/mL) replacing respectively, the cells were continuously incubated for an additional 24 h. Following the kit's instructions, ATP levels were measured. Luminescence was quantified using a luminometer.

1.22 Hemolytic ratios tests

4% sheep blood cells (100 μL) was added to varying concentrations CFGM solutions at 0, 50, 100, 250, 500, and 1000 μg/mL, respectively and incubated at 37℃ for 2 h. The supernatants were collected after centrifuging at 10000 rpm for 2 min. Absorbance was measured at 450 nm with BioTek instrument. For controls, 100 μL 4% sheep blood cells were mixed with either 900 μL of PBS (negative control) or 900 μL of deionized water (positive control). The provided formula: hemolysis rate (%) = $[(D_t\text{-}D_{nc}) / (D_{pc}\text{-}D_{nc})] \times 100\%$ was used for calculating the hemolysis rate.

1.23 Construction of animal models for antitumor efficacy study

An orthotopic breast cancer tumor model was established in 4T1 mice. CFG^{Cy5} (80 μL, 1mg/mL) and $CFG^{Cy5}M$ (80 μL, 1mg/mL) were injected through the tail vein when the tumor volume reached roughly 200 mm^3. In vivo fluorescence was observed using an in vivo imaging system (IVIS Spectrum, Ex 650 nm, Em 670 nm) at 2, 4, 6, 12 and 24 h after injecting.

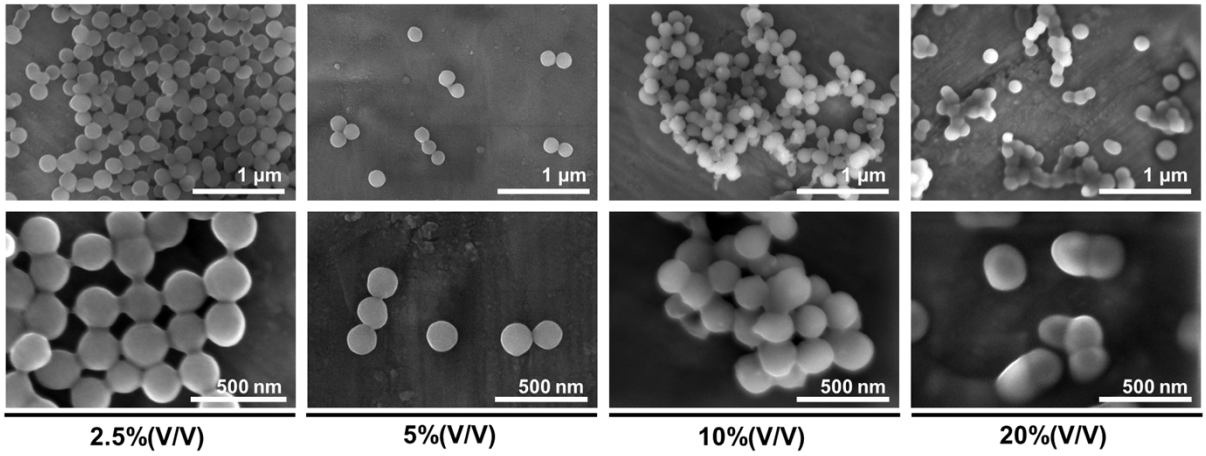
The tumor-bearing mice were randomized into six groups (n=5): Control (80 μL, PBS), COF (80 μL, 1mg/mL), CF (80 μL, 1mg/mL), CG (80 μL, 1mg/mL), CFG (80 μL, 1mg/mL), and CFGM (80 μL, 1mg/mL) when the tumor volume reached roughly 100 mm^3. Over 21-day period, the volume of the tumor and the body weight of the mice were recorded every other day. The formula: $1/2 \times L \times W \times H$ was used for calculating tumor volume. Upon the tumor volume reaching roughly at 100 mm^3, the mice were randomly divided into six groups (n=3) and treated with different materials over a 50-day period.

1.24 Statistical analysis

One-way ANOVA using the Tukey post-test was used for calculating statistical significance (*$p < 0.05$, **$p < 0.01$, *** < 0.001, ****$p< 0.0001$).

206

207 **Figure S1.** TEM images of Fe_2O_3 nanoparticles. No clear lattice fringes were observed, indicating the quasi-
208 amorphous structure of the Fe_2O_3 nanoparticles.



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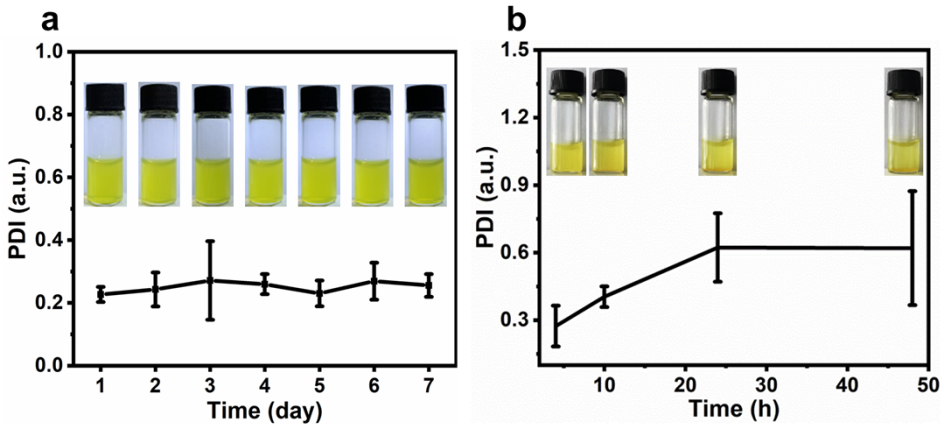
210 **Figure S2.** SEM images of COF synthesized under the acetic acid catalyst with different concentrations. At
211 concentration of 5% (V/V), the as-synthesized COF possessed uniformed morphology of nanospheres.



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213 **Figure S3.** Polydisperse index of (a) COF in water for 7 days and (b) CFGM in water for 48 h. (inset: photos of
214 COF in water for 7 days and CFGM in water for 48 h). COF was dispersed in water without sedimentation within 7
215 days, indicating that it had advanced water dispersibility. After wrapping GOx and Fe_2O_3, although the water
216 dispersibility decreased, it could still maintain no sedimentation for a long time (48 h).

Figure S4. FTIR spectra of different nanoparticles. The obvious C=N stretching vibration at 1618 cm^{-1} for COF suggested its formation via Schiff base reaction between aldehyde group from DMTP and amino group from TAPB.

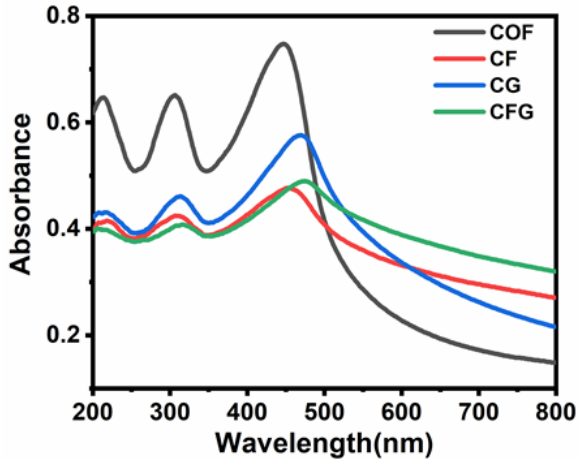

Figure S5. UV-vis spectra of different nanoparticles. After Fe_2O_3 and GOx loading, the adsorption at 447 nm of COF had a gradual red-shift.

Figure S6. CLSM images of CFG and CFGM treated with DiO probe.

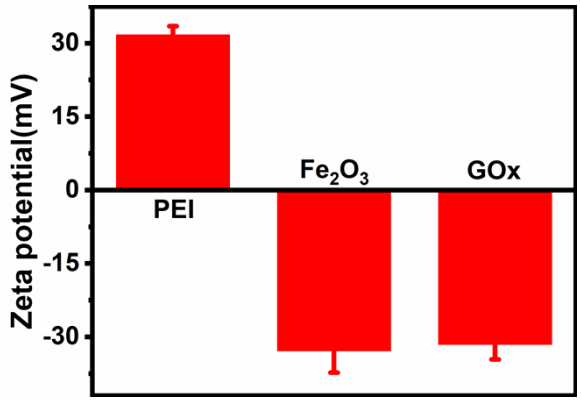

Figure S7. Zeta potentials of PEI, Fe_2O_3 and GOx. Due to the adsorption of a layer of PEI on the COF surface, GOx can be loaded through electrostatic interactions between the cationic PEI and the anionic GOx, as well as through reactions between the PEI amino groups and the carboxyl groups activated on GOx.

Figure S8. Hydrodynamic diameters of different nanoparticles measured via DLS. Because of the hydration, the particle sizes were a little larger than those observed by TEM images [1]. The gradually increased hydrodynamic diameters from COF to CFG might be attributed to the Fe_2O_3/GOx loading and the enhanced hydration.

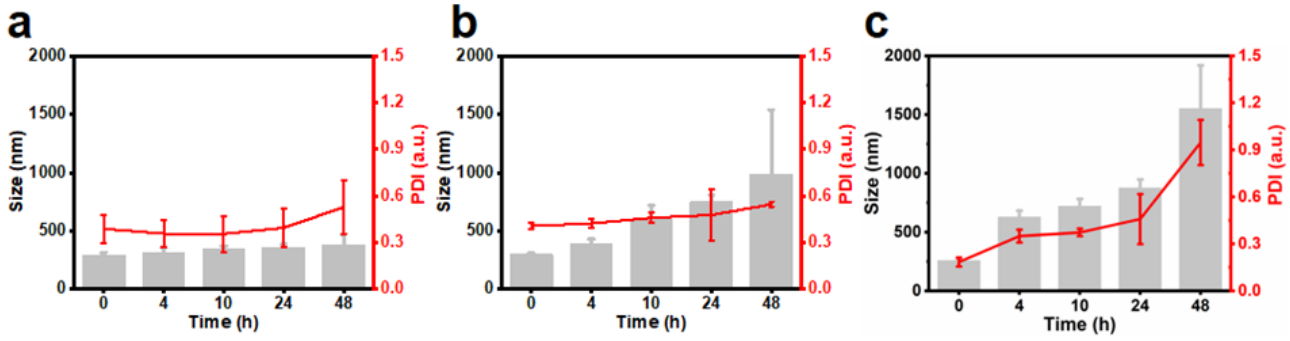

Figure S9. Hydrodynamic diameters and polymer dispersity index of CFG treated in (a) pH 7.4, (b) pH 6.0 and (c) pH 5.0 solution for different time periods. Gradually increasing particle size and PDI suggested the nanoparticles were gradually integrated together to form large congeries possibly due to the degradation fusion. CFG remains stably dispersed at pH 7.4. At pH 6.0, there is a slight increase in PDI and particle size, suggesting possible degradation and formation of aggregates. At pH 5.0, aggregation is even more pronounced.

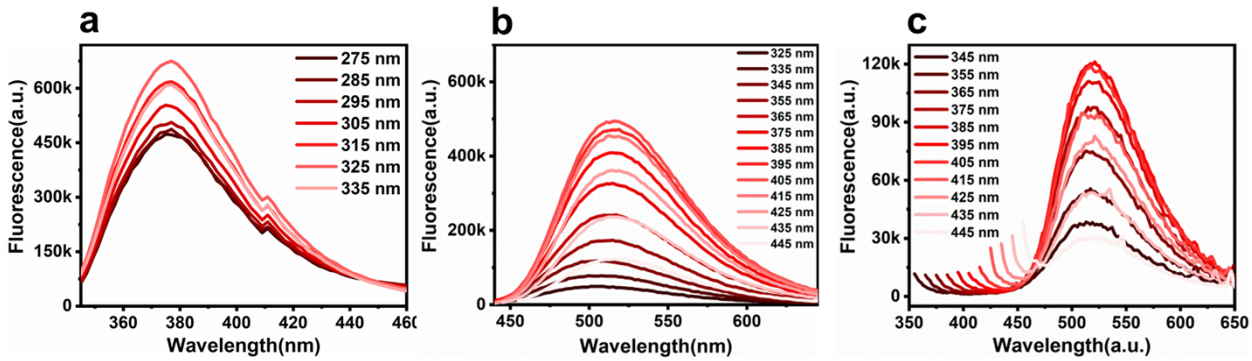

Figure S10. Fluorescence spectra of (a) TAPB, (b) DMTP and (c) COF excited at different excitation wavelengths. The COF inherited the excitation-independent FL emissions from DMTP.

Figure S11. The remaining glucose concentrations in glucose solution (5 mM) (a) after reaction with varied concentrations of CG for different time periods. (b)after reaction with CFG treated with pH 5.0 for different time periods. The consumption rate of 5 mM glucose after 24 h of CFG degradation was found no significant reduced as compared to the glucose consumption rate of undegraded CFG.

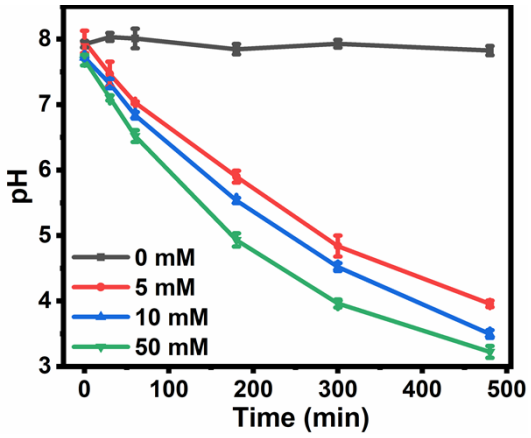

Figure S12. The pH values of the glucose solution with varied concentrations after reaction with CFG (200 μg/mL) for different time periods.

Figure S13. (a-c) UV-vis spectra of the TMB-contained solution for probing ROS generation after reaction with different concentrations of CF in the presence of H_2O_2 under the varied pHs. (d) UV-vis spectra of TMB at 652 nm for probing the • OH generation in glucose solution (5 mM)-contained solution after reacting for 30 min with CFG that was treated at pH 5.0 for different time periods. as compared to that of undegraded CFG, the ·OH generation after CFG degradation had a slight reduction, possibly due to the dissolution of Fe_2O_3 nanoparticles.

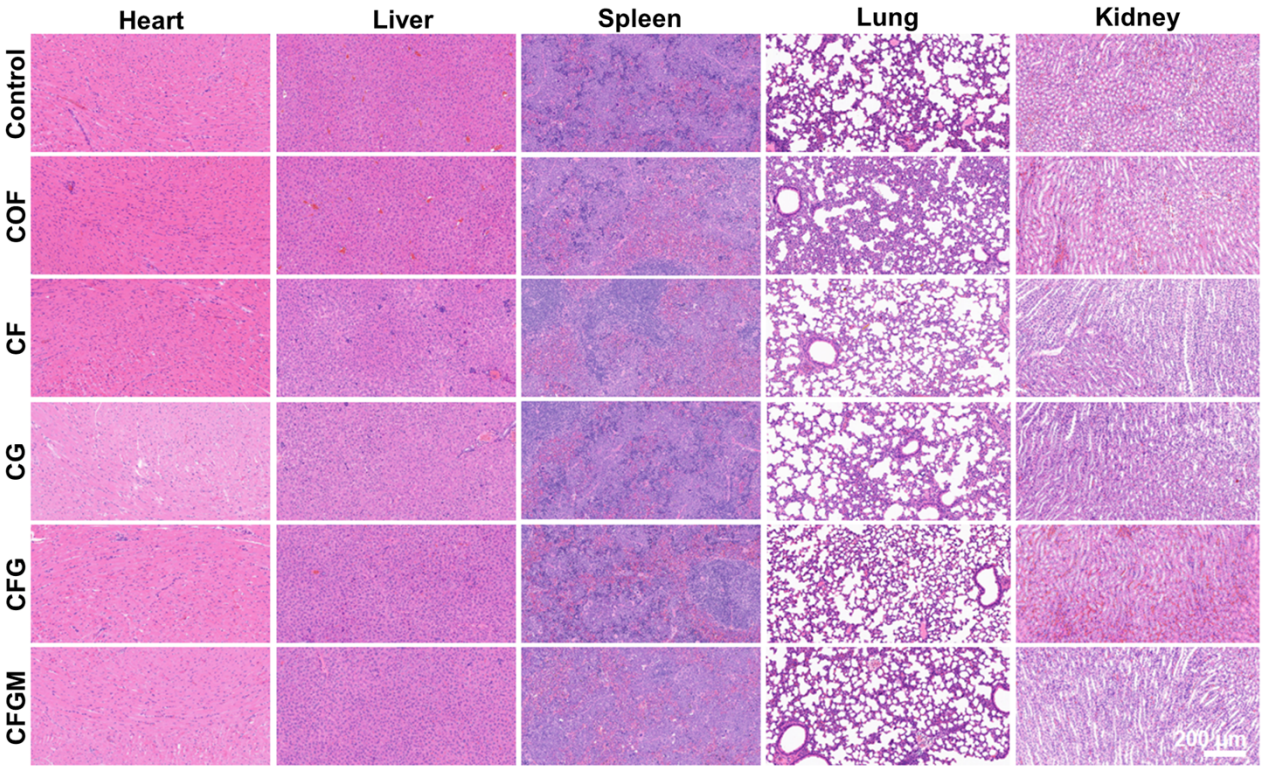

Figure S14. H&E images of heart, liver, spleen, lung and kidney tissues from the 4T1 tumor-bearing mice on the 21st day after various treatments.

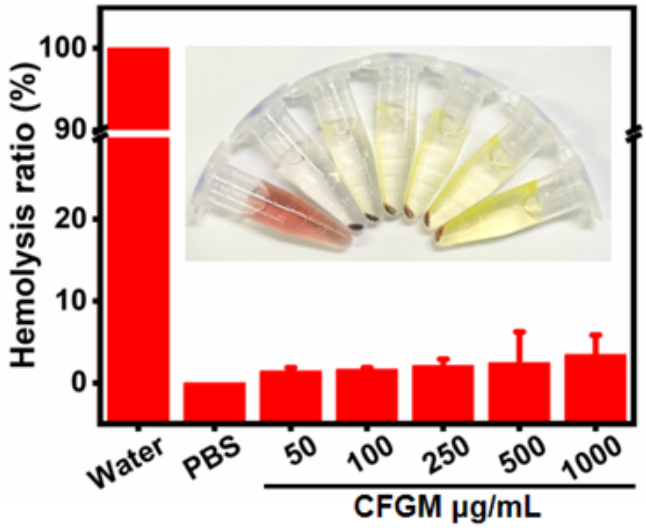

Figure S15. Hemolytic ratios of CFGM with different concentrations. The inset is the photos of supernatants.

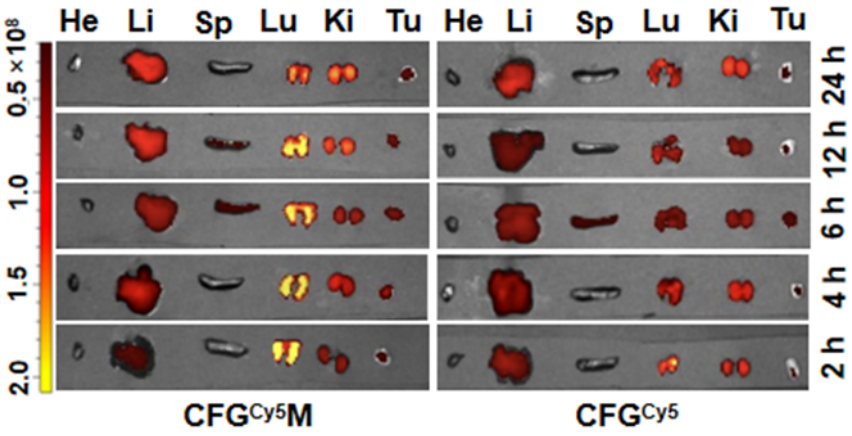

Figure S16. Ex vivo tissue fluorescence images of tumor-bearing mice after intravenous injection with CFG^{Cy5} and $CFG^{Cy5}M$ at different time points.

[1] S. Chen, J. Duan, X. Xie, Y. Fu, M. Zi, Adsorption enhances CH4 transport-driven hydrate formation in size-varied ZIF-8: Micro-mechanism for CH4 storage by adsorption-hydration hybrid technology, Chem. Eng.J. 482 (2024) 148957.