Supplementary Information

An intelligent DNAzyme theranostic probe for *in situ* FTO bioimaging and real-time therapeutic efficacy evaluation in living cells

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1. Reagents and Materials

Glucose oxidase (GOD), sodium borohydride ($NaBH_4$) and ammonium iron (II) sulfate hexahydrate ($(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$) were purchased from Sigma-Aldrich. Zinc nitrate hexahydrate ($Zn(NO_3)_2 \cdot 6H_2O$), nitrogen-containing ligands (2-aminobenzimidazole, 2-methylimidazole) and polymeric dispersant PVP (MW = 10,000) were purchased from Shanghai Aladdin Biochemical Technology (China). HCl, NaOH, H_2O_2 and succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC) were purchased from Shanghai Macklin Biochemical Co., Ltd (China).

All DNAs utilized in this study were purchased from Shanghai Sangon Biological Engineering Technology & Services (China) with their sequences listed in Table S1. The human embryonic kidney 293 cells (HEK-293T) and human breast cancer cells (MCF-7) were supplied by Shanghai Cell Bank of the Chinese Academy of Sciences. The reactive oxygen species fluorescent probe (DCFH-DA), live/dead cell staining kit (Calcein-AM/PI), glucose activity assay kit, and hydrogen peroxide content assay kit were all purchased from Solarbio Science & Technology Co., Ltd. (Beijing, China). Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were purchased from Corning (USA). Ultrapure water (resistance >18 MΩ cm) was obtained by using a Milli-Q reference system (Billerica, MA, USA).

2. Instrumentation

The microstructure and morphology of GOx-Fe^0@ZIF-8-NH_2 were characterized by using a scanning electron microscopy (SEM, US-70, Hitachi). Dynamic light scattering (DLS) analysis was performed by using a Malvern Zeta Sizer Nano ZS90 (London, UK). The fluorescence measurements were recorded by using a RF-6000 spectrophotometer (Shimadzu, Japan). Detection of Zn^{2+} and Fe^{2+} was performed by using an Inductively Coupled Plasma Mass Spectrometry (ICP-MS, Agilent 7800, US). Fluorescence measurements were recorded by using a RF-6000 spectrophotometer (Japan). The CCK-8 was utilized for cell quantification by the Varioskan Flash microplate reader from Thermo Fisher Scientific (China) at 450 nm. Confocal fluorescence images of cells were obtained by using a FV3000 laser-scanning confocal microscopy (Olympus, Japan). The T20S Series Thermal Cycler for Porous Plates was purchased from Long Gene (China) and the Fire-Reader V10 Gel Imager was purchased from UVITEC (UK).

3. Methods

3.1. Denaturing Polyacrylamide Gel Electrophoresis (PAGE) Assay

The demethylation of m^6A-E-strands was first performed in a 100 μL reaction system containing 50

mM HEPES (50 mM HEPES, 100 mM NaCl, pH = 7.4), 1 μM m^6A-E-strands, FTO protein (250 nM), 283 μM ammonium iron (II) sulfate hexahydrate, 300 μM α-ketoglutarate, 2 mM L-ascorbic acid, and 50 μg/mL bovine serum albumin (BSA). The reaction mixture was incubated at 37 °C for 3 h. Subsequently, the process was terminated by adding 5 mM EDTA, followed by heating at 95 °C for 5 min. Then, the mixture containing FTO-treated m^6A-E-strands (200 nM), S-strands (1 μM), and Zn^{2+} (200 μM) was prepared in HEPES buffer (50 mM HEPES, 100 mM NaCl, pH = 7.4) and incubated at 37 °C for 3 h. After incubation, 10 μL of the reaction mixture was combined with 2 μL of loading buffer and loaded onto 12% denaturing polyacrylamide gel. Electrophoresis was carried out in 1× TBE buffer at a constant voltage of 100 V for 1 h. Following electrophoresis, the gel was stained with GelRed nucleic acid dye. Fluorescence imaging was performed using a Fluor Chem FC3 imaging system (Protein Simple, USA) under 365 nm UV excitation.

3.2. Encapsulation Verification by Protease Digestion

To verify the encapsulation of GOx within the ZIF-8 framework, free GOx and The GOx-Fe^0@ZIF-8-NH_2 were each incubated with proteinase K (final concentration 1 mg/mL) in HEPES buffer (50 mM HEPES, 100 mM NaCl, pH = 7.4) at 37 °C for 1 h. Untreated free GOx and The GOx-Fe^0@ZIF-8-NH_2 incubated without proteinase K under the same conditions served as controls. After incubation, the GOx-Fe^0@ZIF-8-NH_2 samples were centrifuged at 10,000 rpm for 10 min, and the pellets were washed twice with HEPES, then resuspended in fresh buffer. For free GOx samples, the reaction mixtures were used directly. Following the reaction, the samples were collected and analyzed for glucose oxidase activity using glucose oxidase activity test kit.

3.3. Fluorescence Detection of Active FTO in solution

For fluorescence assay, 200 μL reaction mixture was prepared in HEPES buffer (50 mM, 100 mM NaCl, pH = 7.4), containing 100 nM m^6A-DNAzyme (pre-treated with FTO) and GOx-Fe^0@ZIF-8-NH_2+S-substrates. Fluorescence signals were acquired using RF-6000 fluorescence spectrophotometer. Emission spectra from 560 to 700 nm were recorded under an excitation wavelength of 540 nm. Additionally, time-dependent maximum change in fluorescence intensity were monitored at a fixed emission wavelength of 565 nm.

To verify the specific recognition capability of the constructed detection system for FTO protein, experiments were performed as follows. First, a series of 100 μL reaction systems were prepared. Each contained the basic components required for the demethylation reaction: 50 mM HEPES buffer (pH =

7.4), 1 μM m^{6}A-modified DNAzyme strand (m^{6}A-E strand), 283 μM $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$, 300 μM α-ketoglutarate, 2 mM L-ascorbic acid, and 50 μg/mL BSA. Subsequently, different testing proteins and small molecules were added to each reaction system, specifically including: 250 nM FTO protein (positive control group), 200 nM thrombin, 200 nM immunoglobulin G (IgG), 50 μg/mL BSA, and 200 nM glucose oxidase (GOD). A blank control group without any protein or small molecule was also set up. All reaction systems were incubated at 37 °C for 3 h. After incubation, the reaction was terminated by adding 5 mM EDTA, followed by heating at 95°C for 5 min. Then, the above solution was incubated with GOx-Fe^0@ZIF-8-NH_2+S-substrates at 37 °C for 0.5 h. After the reaction, the product was collected by centrifugation, washed, and then incubated in HEPES (50 mM HEPES, 100 mM NaCl, pH = 6.5) buffer at 37 °C for 3 h. Finally, the fluorescence recovery signals of each group were detected using a fluorometer, and the specific response capability of the detection system to FTO protein was evaluated by comparing the fluorescence intensities of the positive control group with those of each test group.

3.4. Fenton Interference Control Experiments

To exclude potential interference from the Fenton reaction, the following five groups were compared under identical conditions (HEPES buffer, 50 mM HEPES, 100 mM NaCl, pH = 6.5, 37 °C): (1) free m^{6}A-DNAzyme; (2) free m^{6}A-DNAzyme+Fe^{2+} (5 μM) + H_2O_2 (2 mM); (3) GF@ZD; (4) GF@ZD+H_2O_2 (2 mM); (5) GF@ZD+FTO (250 nM). After incubation for 3 h, the fluorescence intensity of Cy3 (λ_{ex} = 540 nm, λ_{em} = 560 ~ 700 nm) was recorded using a fluorescence spectrophotometer.

3.5. Release of pH-Triggered Zn^{2+} and Fe^{2+}

The material was dispersed in 15 mL of PBS buffer solutions at different pH values (5.4, 6.5, 7.4) and continuously incubated at 37 °C under shaking at 100 rpm. At predetermined time intervals (0, 1, 2, 4, 8, 12, 24, and 48 h), samples were collected and centrifuged at 10,000 rpm. 2 mL aliquot of the supernatant was taken for Zn^{2+} and Fe^{2+} concentration measurement. The concentration of ions was quantitatively analyzed using an inductively coupled plasma mass spectrometry (ICP-MS).

3.6. Capability of •OH Generation by GF@ZD in solution

The capacity of GF@ZD to generate hydroxyl radicals (•OH) under varying pH conditions was assessed *in vitro* by monitoring the degradation of methylene blue (MB). The reaction system was established by dispersing the nano-system in PBS buffers at different pH values (5.4, 6.5, 7.4), followed by the addition of 10 μg/mL MB and 40.0 mM H_2O_2. The extent of •OH-induced oxidative degradation was evaluated using an ultraviolet-visible (UV-Vis) spectrophotometry by measuring the decrease in the characteristic absorption peak of MB at 660 nm. The absorbance at 660 nm was continuously recorded

over 60 min reaction period to plot the degradation kinetics, thereby providing an analysis of the •OH generation dynamics.

3.7. Catalytic Performance of GF@ZD

To assess the effect of glucose concentration on the catalytic performance of GF@ZD, the GF@ZD (10 μg/mL) was incubated with a series of glucose solutions at varying concentrations (0, 200, 400, 600, 800, and 1000 μg/mL). Following the reaction, the samples were collected and analyzed for glucose oxidase activity and hydrogen H_2O_2 production using assay kits (a glucose oxidase activity test kit and a H_2O_2 assay kit), respectively.

3.8. Cell Culture

MCF-7 cells and HEK-293T cells were cultured in DMEM supplemented with 10.0% fetal bovine serum and 1.0% penicillin-streptomycin, and maintained in a humidified incubator at 37 °C with 5.0% CO_2.

3.9. Cytotoxicity Assessment

MCF-7 cells were seeded in 96-well plates (5.0×10^4 cells/mL, 100 μL/well) and cultured for 24 h. The medium was then replaced with DMEM containing various concentrations (0, 20, 40, 60, 90, and 120 μg/mL) of ZIF-8, GOx-ZIF-8, GOx-Fe^0@ZIF-8-NH_2, or GF@ZD, and the cells were incubated for another 24 h. After washing three times with PBS, 100 μL of DMEM containing 10 μL of CCK-8 reagent was added to each well. Following a 4 h incubation, cell viability was assessed by measuring the absorbance at 450 nm using a microplate reader.

3.10. Intracellular •OH Detection

Intracellular •OH generation was assessed using the DCFH-DA probe. MCF-7 cells were treated with ZIF-8, GOx@ZIF-8, or GF@ZD (90 μg/mL) for 6 h, followed by incubation with 10 μM DCFH-DA for 30 min. After washing with PBS to remove excess probe, fluorescence was visualized by fluorescence microscopy (λ_{ex} = 488 nm, λ_{em} = 500 ~ 600 nm).

4. Supplementary Figures and Table

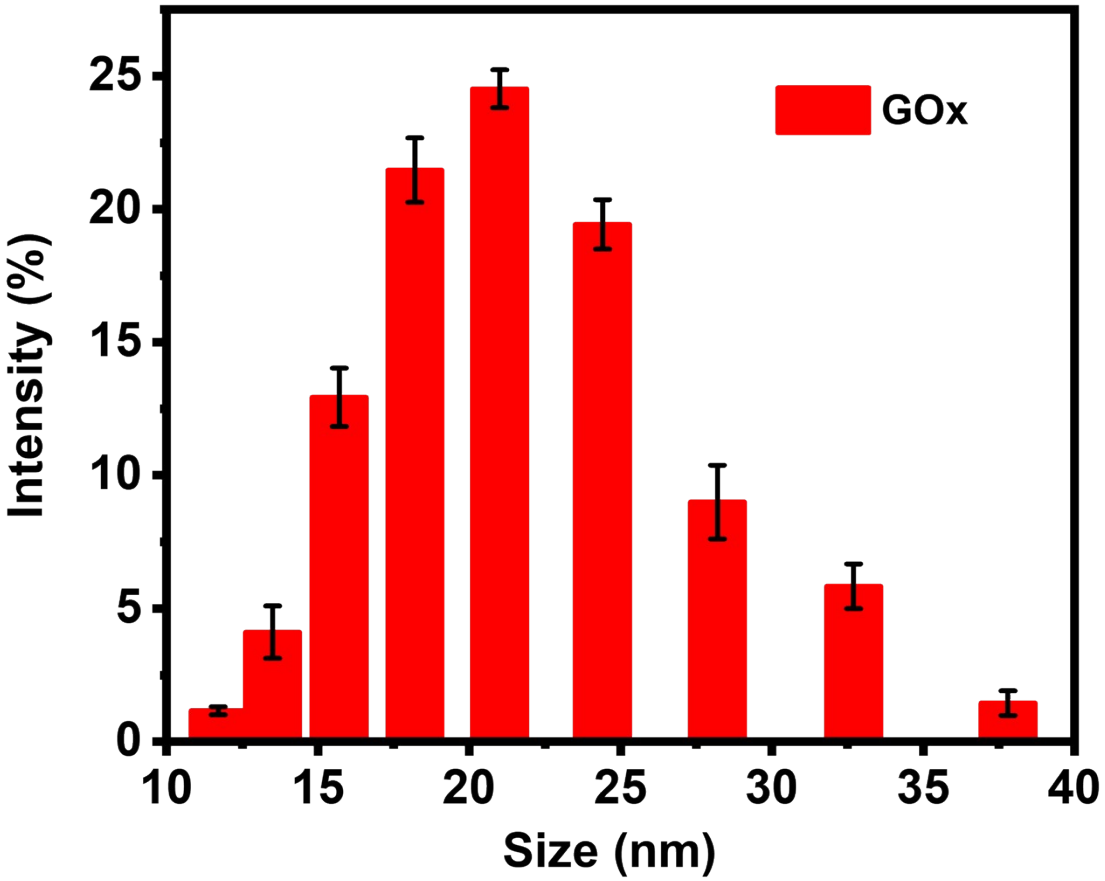

Fig. S1 DLS analysis of GOx; data are represented as means ± SD, n = 3.

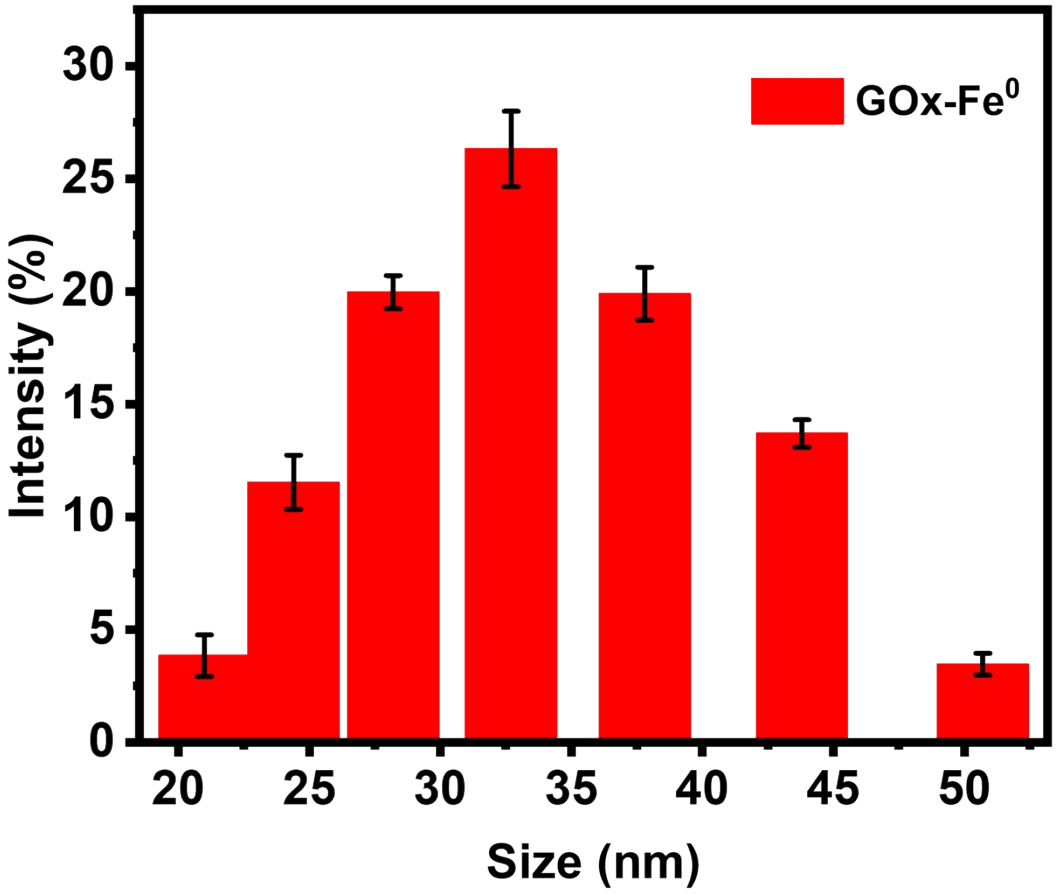

Fig. S2 DLS analysis of GOx-Fe^0; data are represented as means ± SD, n = 3.

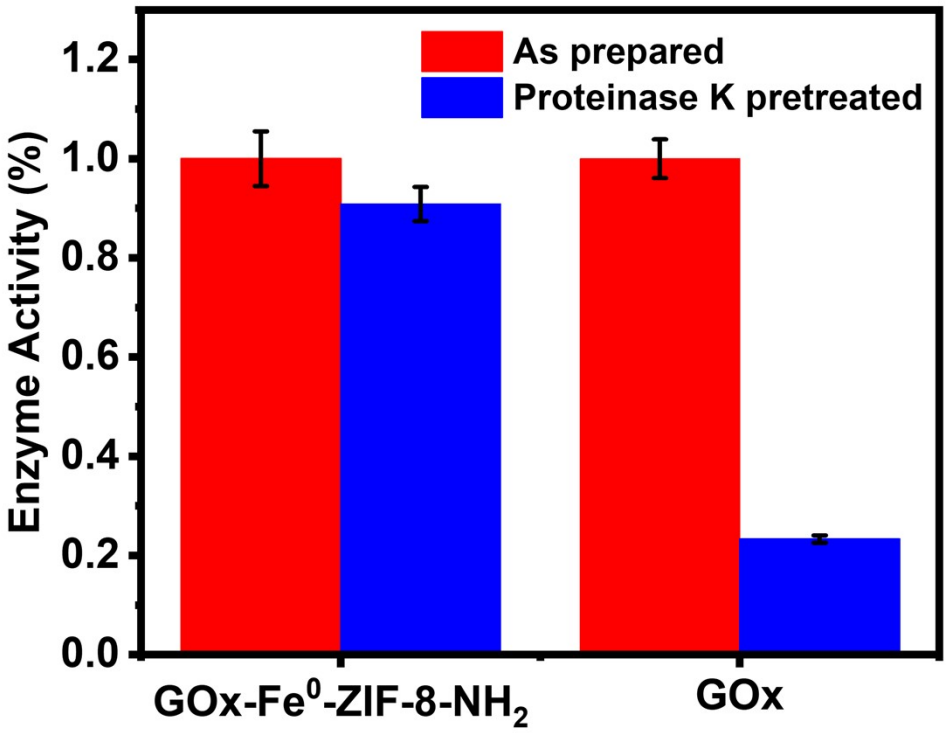

Fig. S3 Encapsulation verification of GOx by protease digestion. Relative enzymatic activity of free GOx and GOx-Fe^0@ZIF-8-NH_2 before and after treatment with proteinase K (1 mg/mL, 37 °C, 1 h). The activity of each untreated sample (as-prepared) was set as 100%; data are represented as means ± SD, n = 3

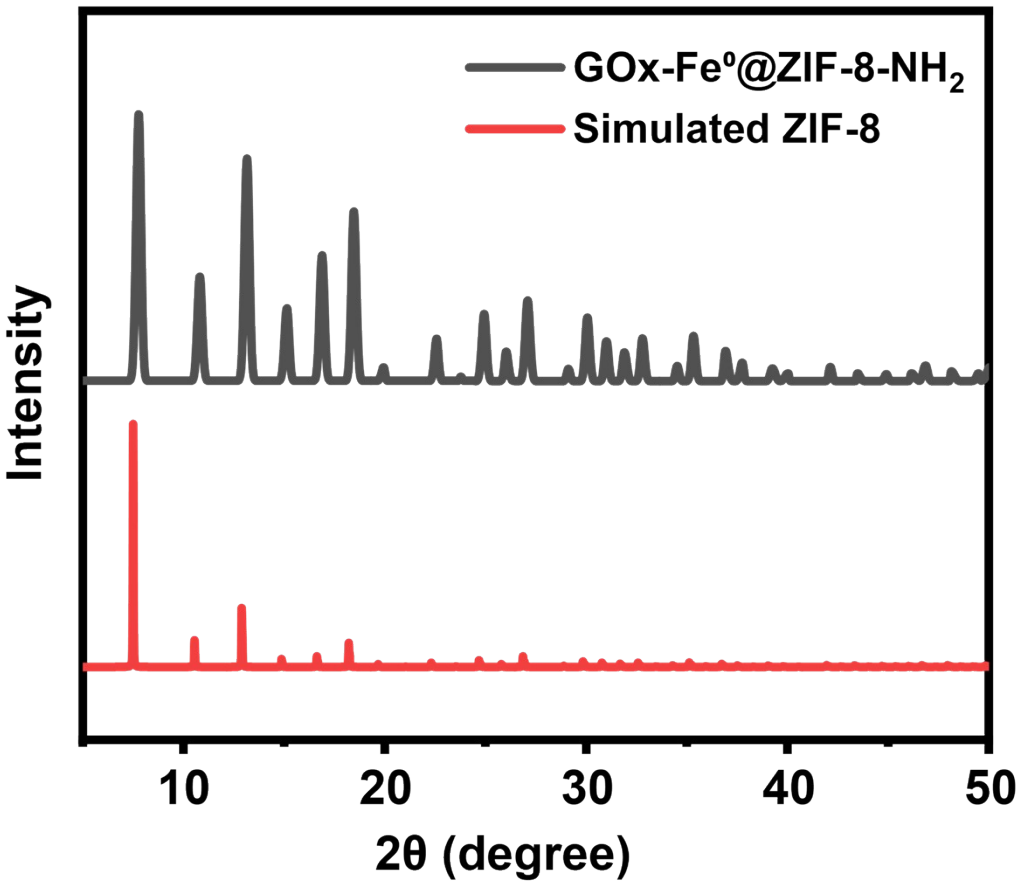

Fig. S4 XRD patterns of GOx-Fe^0@ZIF-8-NH_2 (black), simulated ZIF-8 (red).

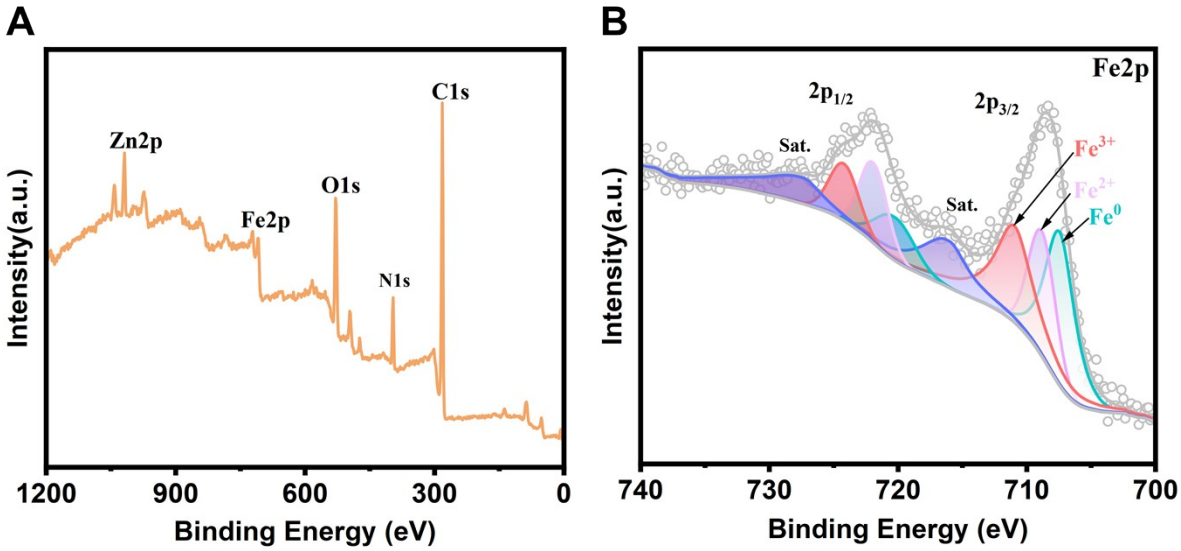

Fig. S5 XPS spectra of the GOx-Fe^0@ZIF-8-NH_2 nanocomposite. (A) The survey spectrum reveals the presence of C, N, O, Fe, and Zn elements. (B) The high-resolution Fe 2p spectrum confirms the existence of Fe^0 species along with Fe^{2+}/Fe^{3+} species resulting from surface oxidation.

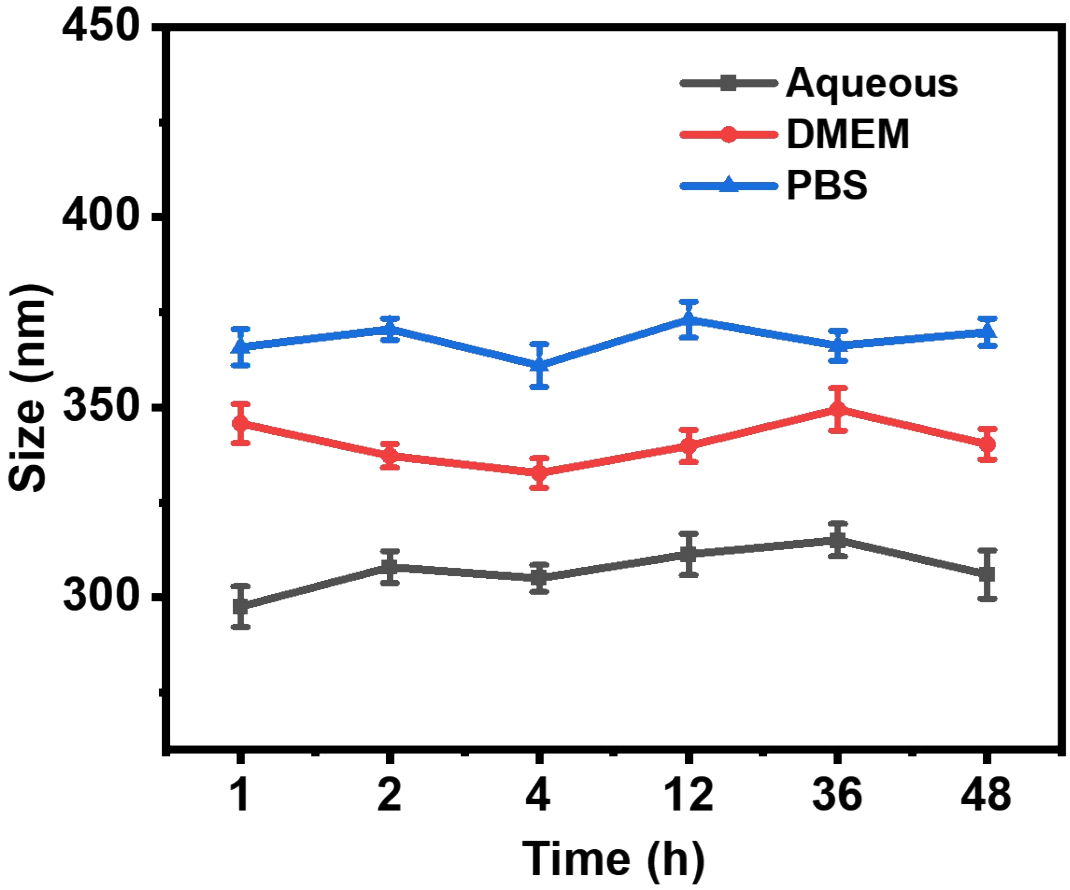

Fig. S6 The stability (sizes change) of the GOx-Fe^0@ZIF-8-NH_2 (1 mg/mL) in different solution (aqueous, DMEM, PBS (10 mM), pH = 7.4); data are represented as means ± SD, n = 3.

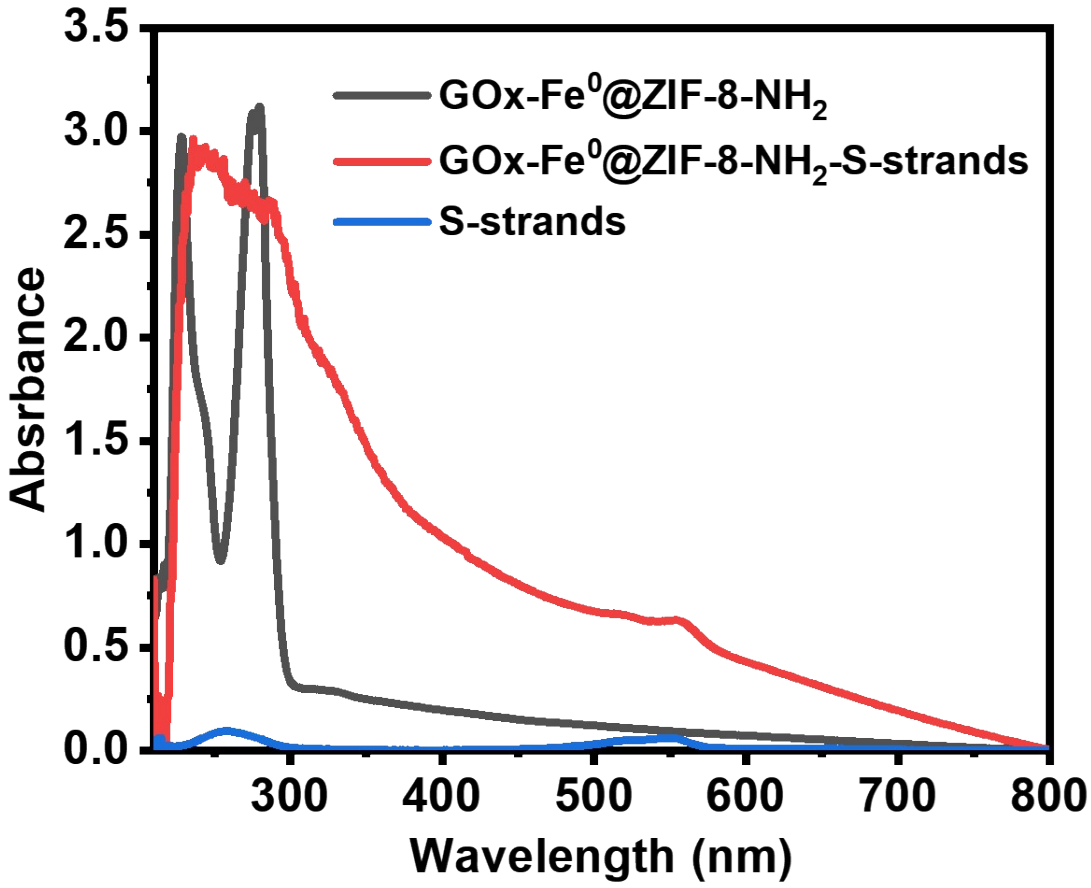

Fig. S7 The UV-vis absorption spectrum of GOx-Fe^0@ZIF-8-NH_2, GOx-Fe^0@ZIF-8-NH_2-S-strands, S-strands.

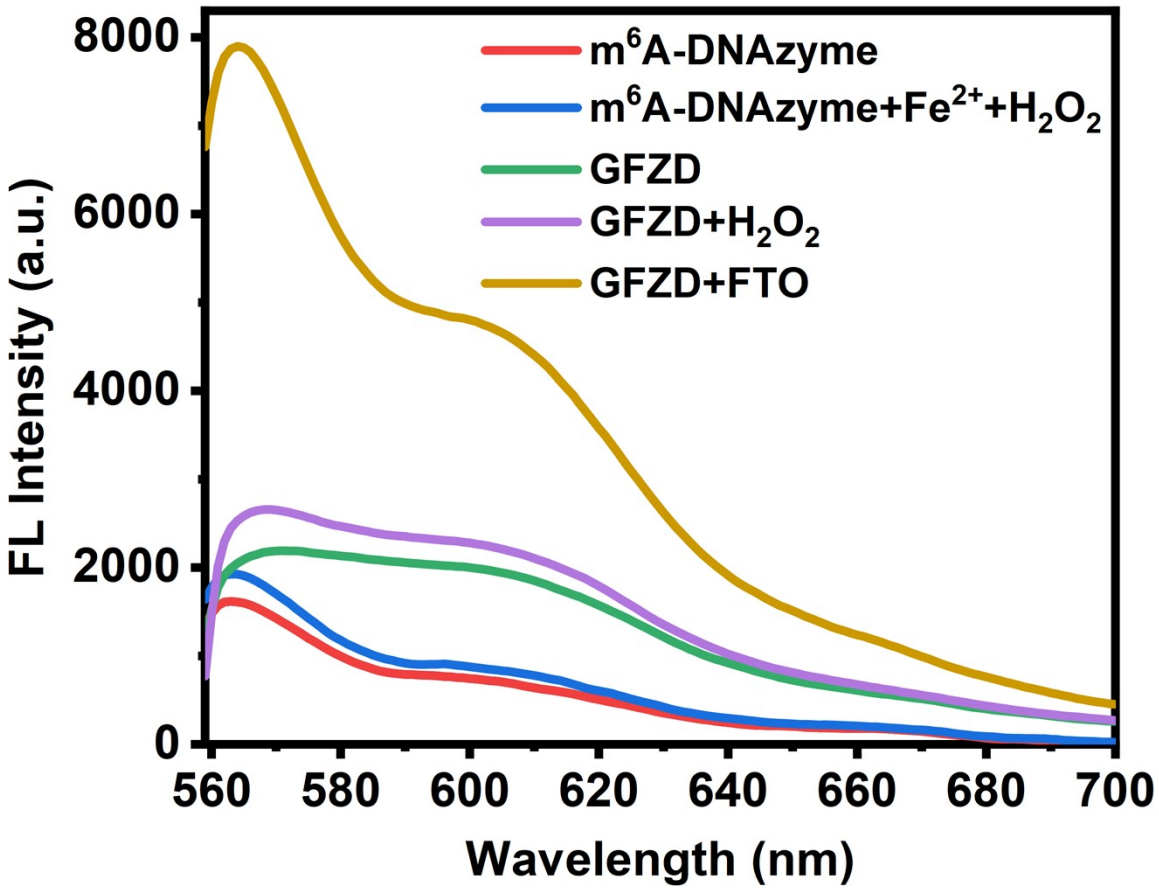

Fig. S8 Exclusion of Fenton reaction interference. Relative fluorescence intensity of Cy3 in different control groups: (1) free m^6A-DNAzyme (red line), (2) free m^6A-DNAzyme+Fe^{2+}+H_2O_2 (blue line), (3) GF@ZD (green line), (4) GF@ZD+H_2O_2, (purple line) and (5) GF@ZD+FTO (yellow line); data are represented as means ± SD, n = 3.

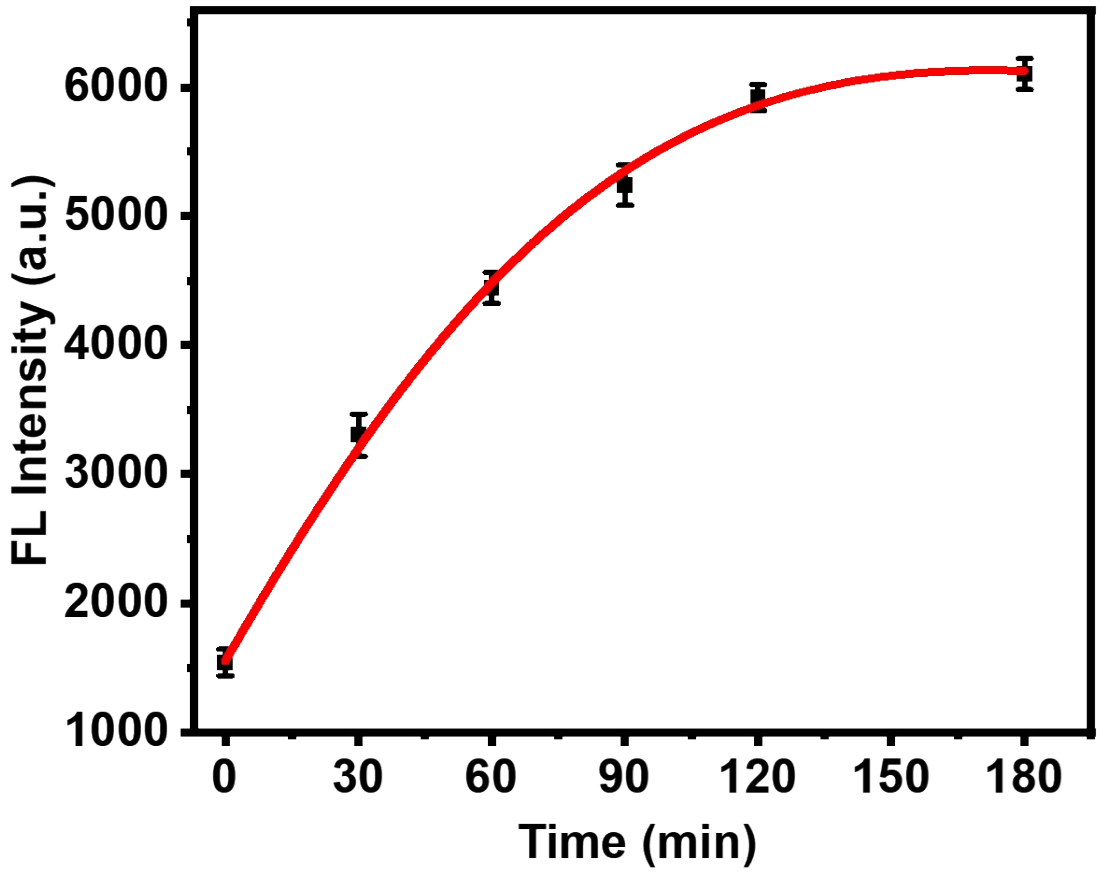

Fig. S9 The fluorescence intensity of GF@ZD (0.125 μg/mL) response to FTO (250 nM) under different time in HEPES buffer (50 mM HEPES, 100 mM NaCl, pH = 6.5); data are represented as means ± SD, n = 3.

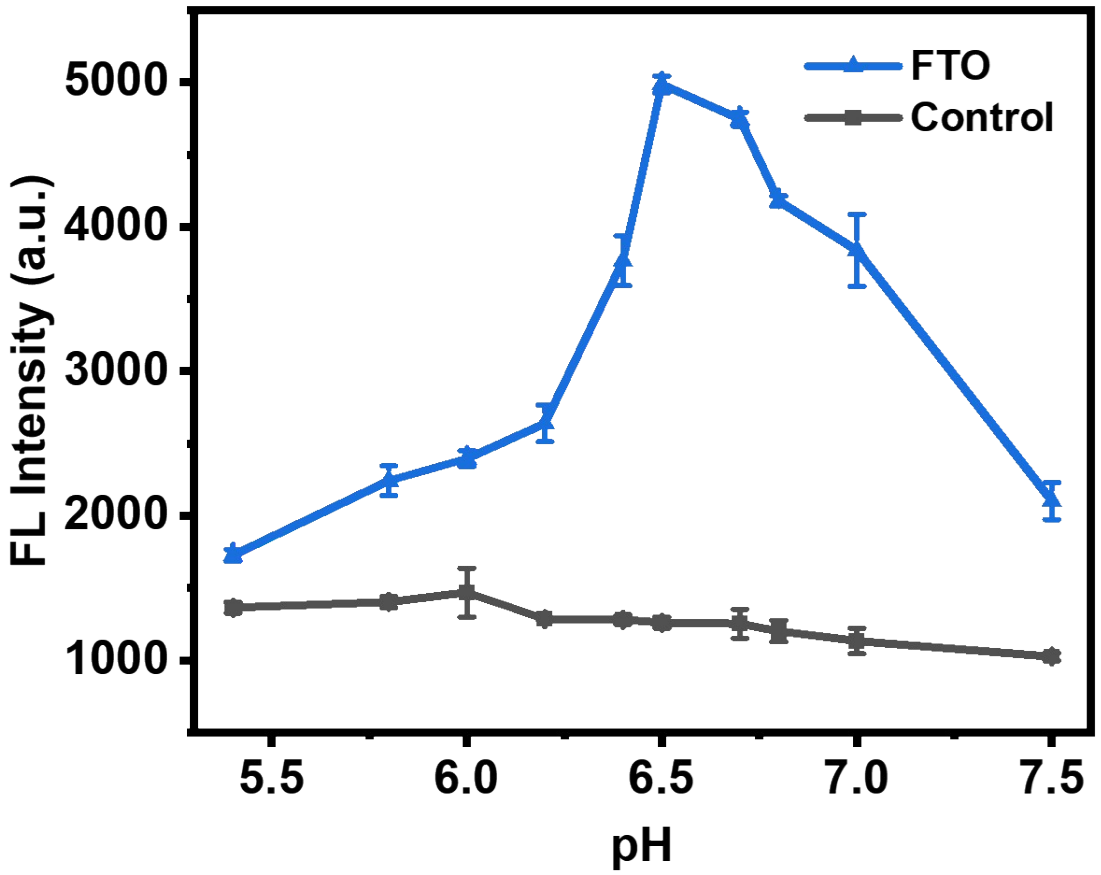

Fig. S10 The fluorescence intensity of FTO treated GF@ZD (0.125 μg/mL) and untreated (Control) for different pH in HEPES buffer (50 mM HEPES, 100 mM NaCl); data are represented as means ± SD, n = 3.

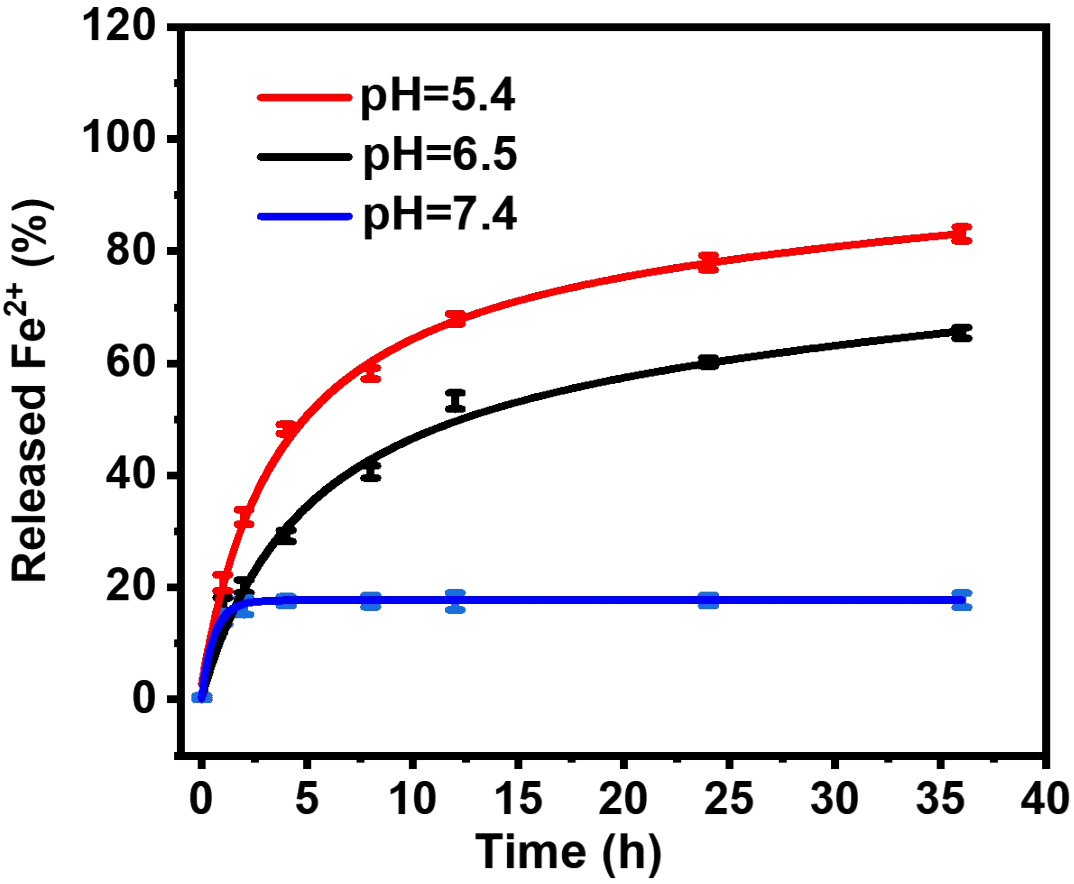

Fig. S11 The release of Fe^{2+} from GF@ZD at different pH by ICP-MS analysis; data are represented as means ± SD, n = 3.

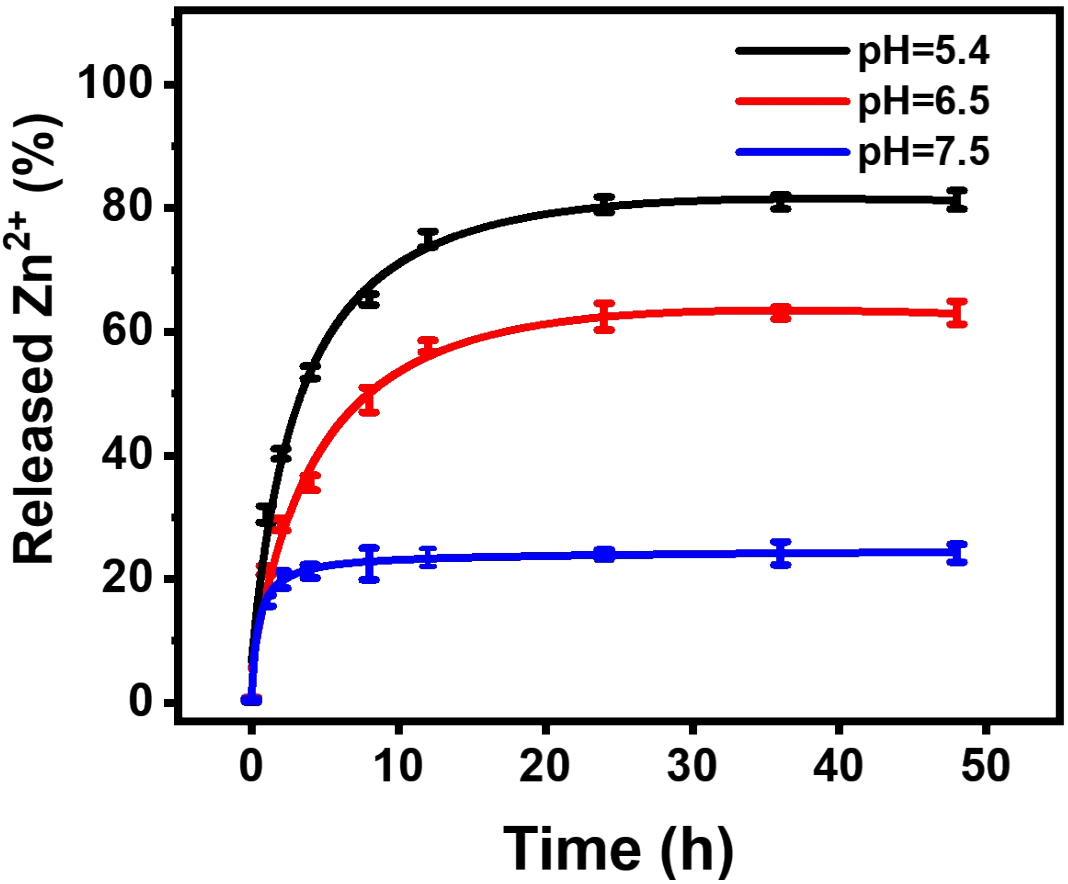

Fig. S12 The release of Zn^{2+} from GF@ZD at different pH by ICP-MS analysis; data are represented as means ± SD, n = 3.

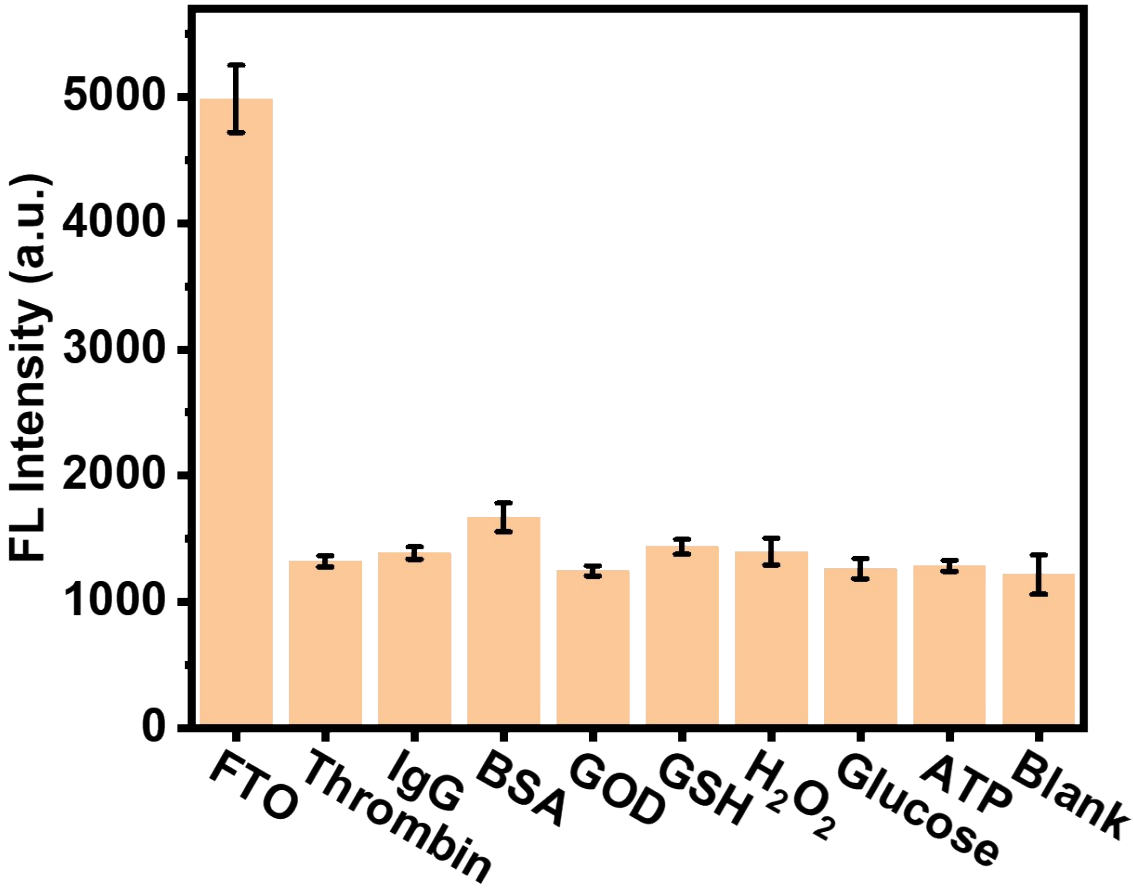

Fig. S13 The histogram of fluorescence intensity of GF@ZD (0.125 μg/mL) response to different species in HEPES buffer (50 mM HEPES, 100 mM NaCl, pH = 6.5), including 250 nM FTO, 200 nM thrombin, 200 nM IgG, 50 μg/mL BSA, 200 nM GOD, 5 mM GSH, 100 μM H_2O_2, 2 mM glucose and 5 mM ATP; $\lambda_{ex=}$ 540 nm, λ_{em} = 560 ~ 700 nm; data are represented as means ± SD, n = 3.

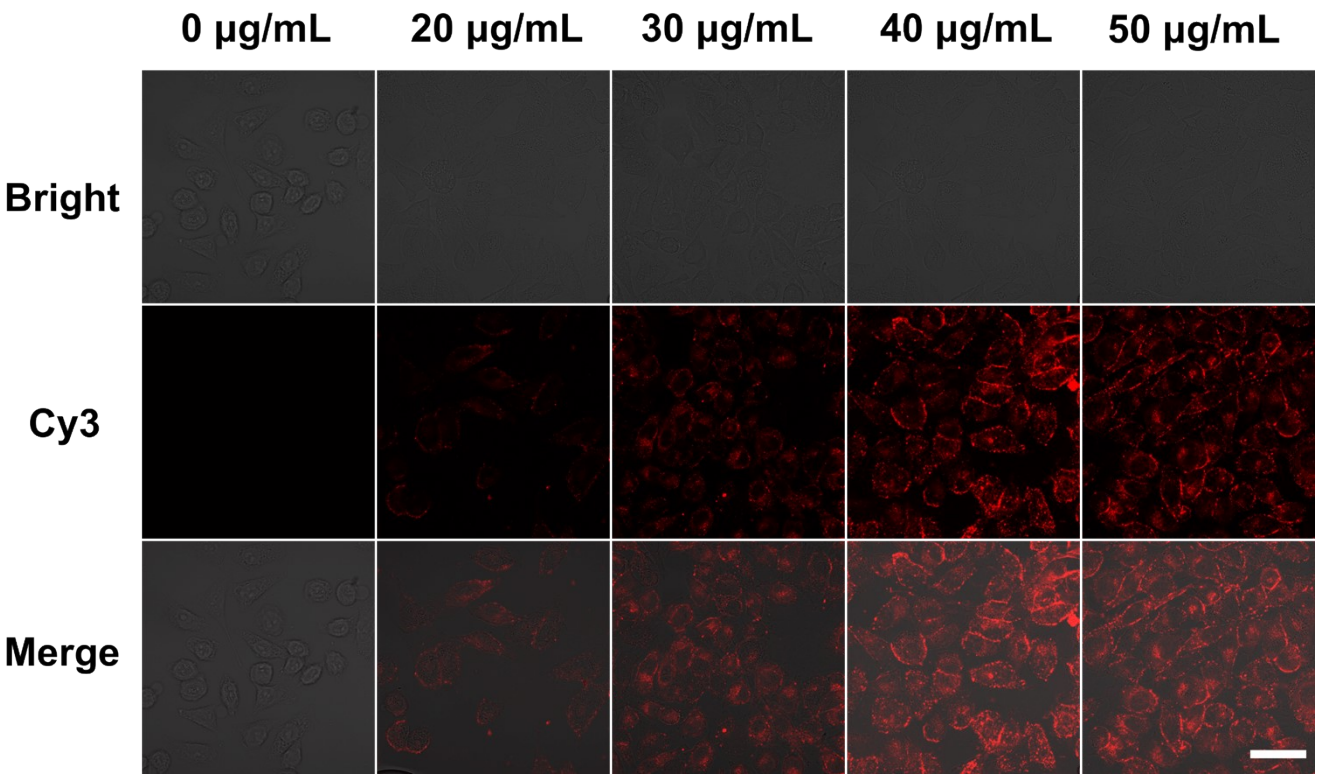

Fig. S14 Confocal fluorescence bioimaging of MCF-7 cells with GF@ZD for various concentration at 37 ℃ for 6 h; λ_{ex} = 561 nm; λ_{em} = 570 ~ 620 nm; the scale bar is 50 μm.

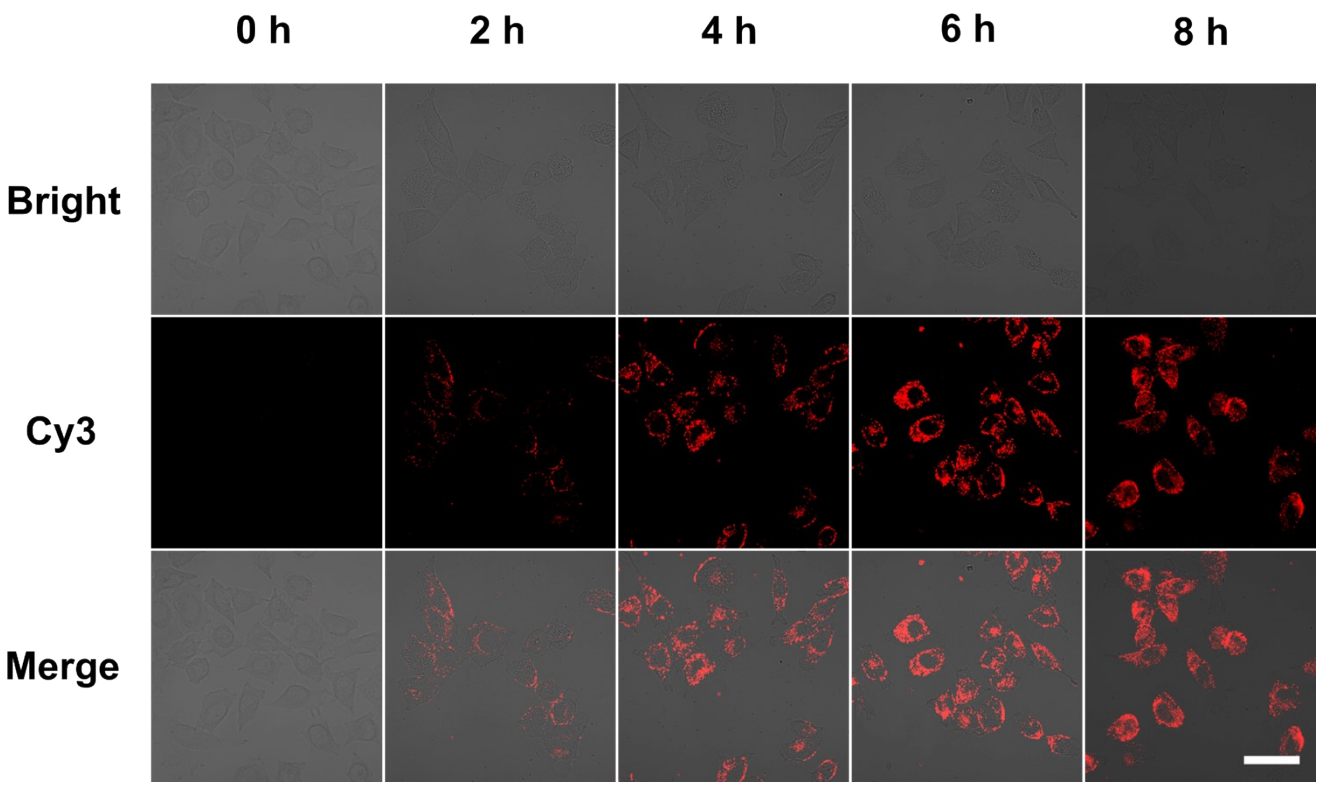

Fig. S15 Confocal fluorescence bioimaging of MCF-7 cells with GF@ZD (50 nM) at various time at 37 °C; λ_{ex} = 561 nm; λ_{em} = 570 ~ 620 nm; the scale bar is 50 μm.

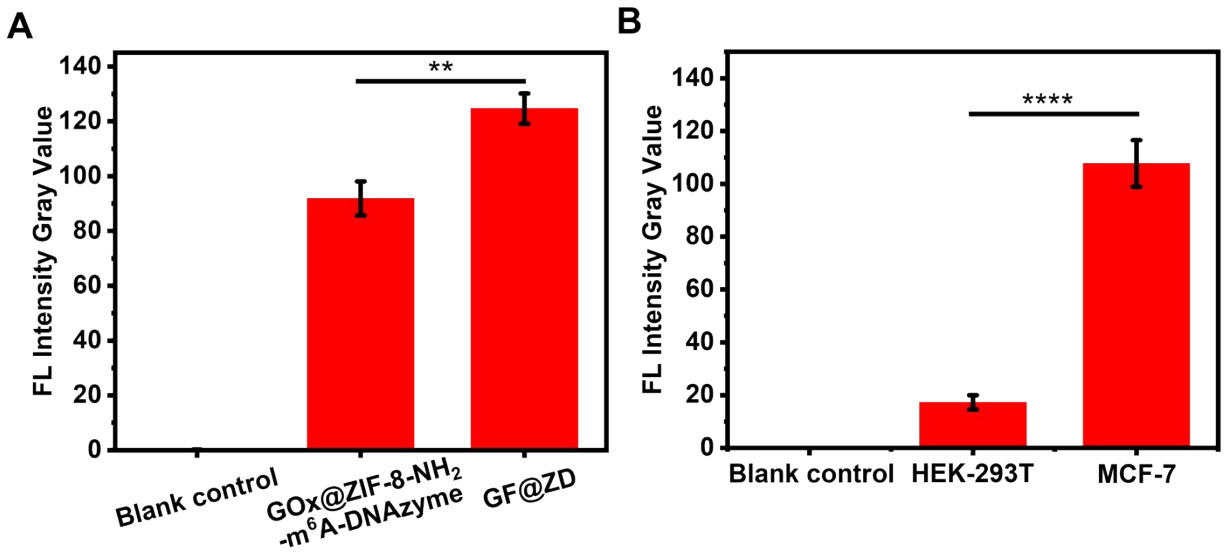

Fig. S16 (A) Histogram of the corresponding fluorescence intensity values in Fig. 4 with ImageJ software. (B) Histogram of the corresponding fluorescence intensity values in Fig. 5 with ImageJ software. Data are represented as means ± SD, n = 3. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

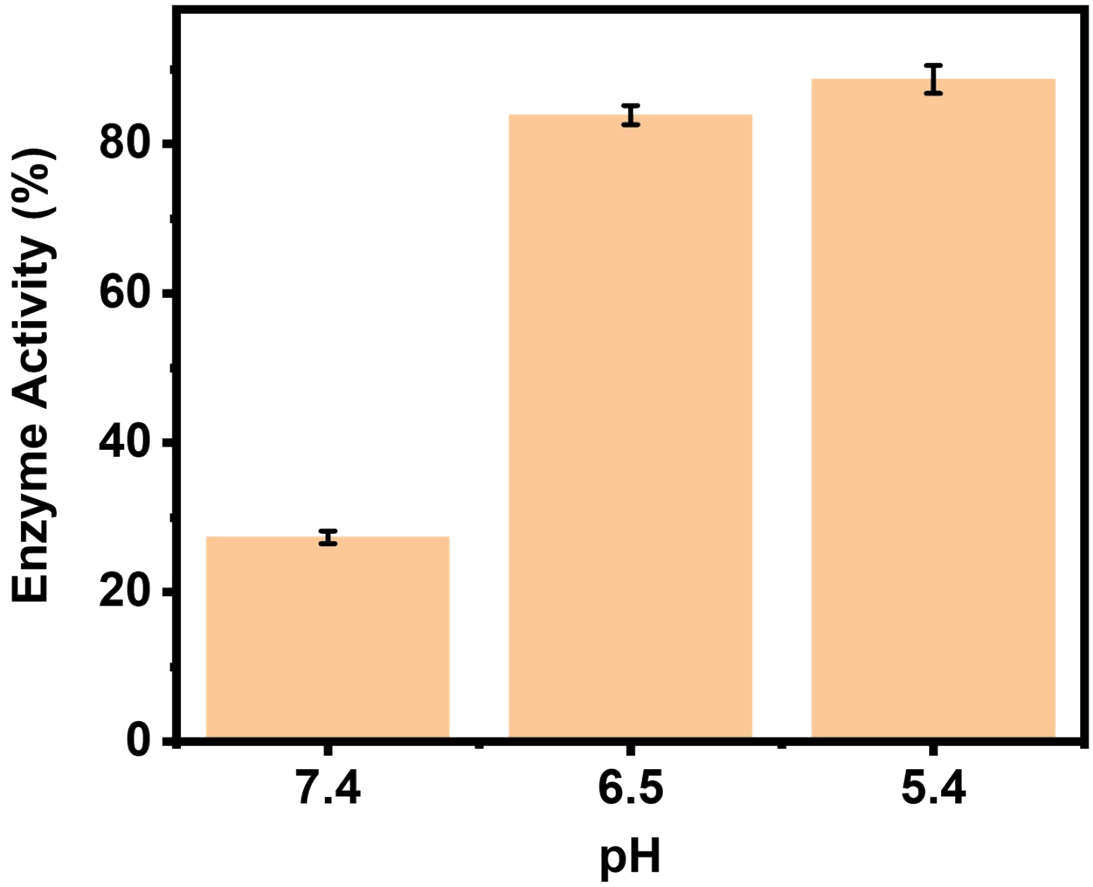

Fig. S17 The relative enzymatic activity of GOx from GF@ZD at different pH by glucose oxidase activity test kit; data are represented as means ± SD, n = 3.

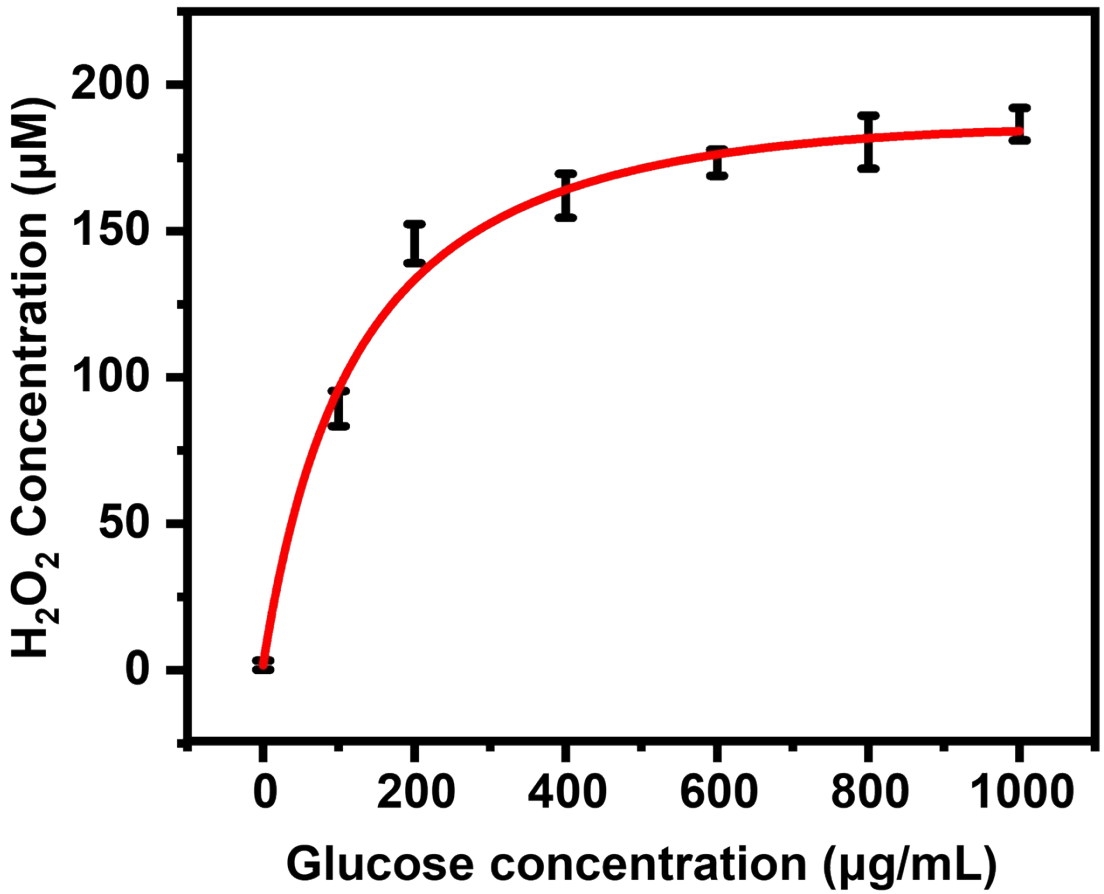

Fig. S18 Variation of the H_2O_2 concentration of GF@ZD after 4 h of incubation with different concentrations of glucose by H_2O_2 assay kit; data are represented as means ± SD, n = 3.

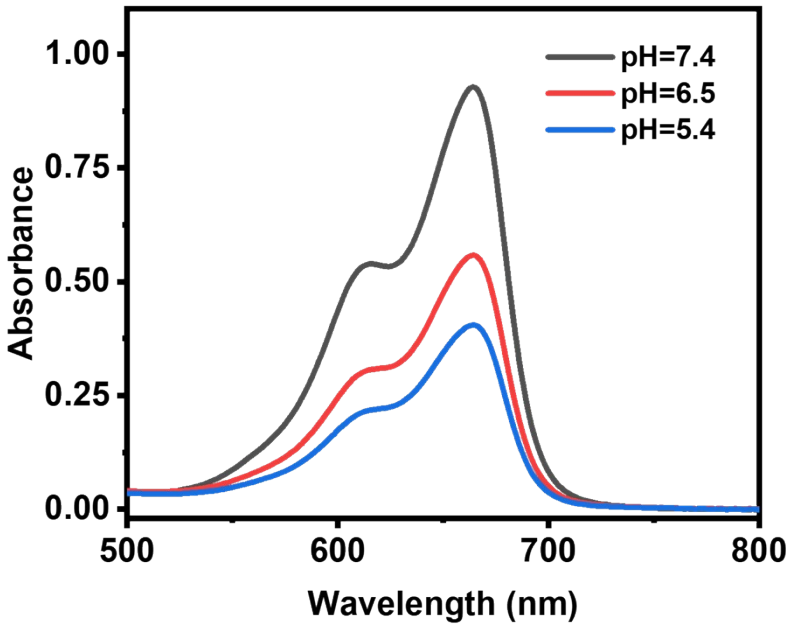

Fig. S19 The UV−vis absorption spectrum of •OH generated from methylene blue degradation at different pH by GF@ZD.

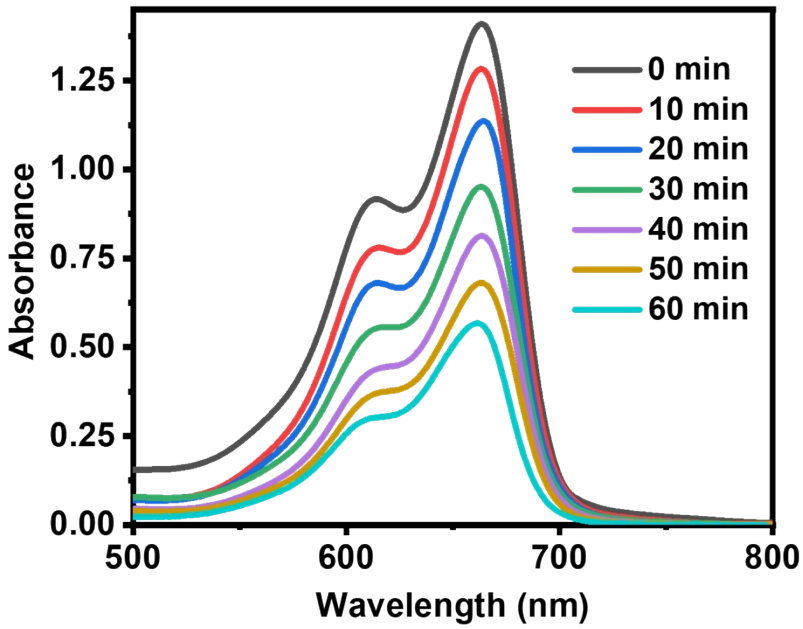

Fig. S20 The UV−vis absorption spectrum of •OH generated from methylene blue degradation by GF@ZD for different time periods.

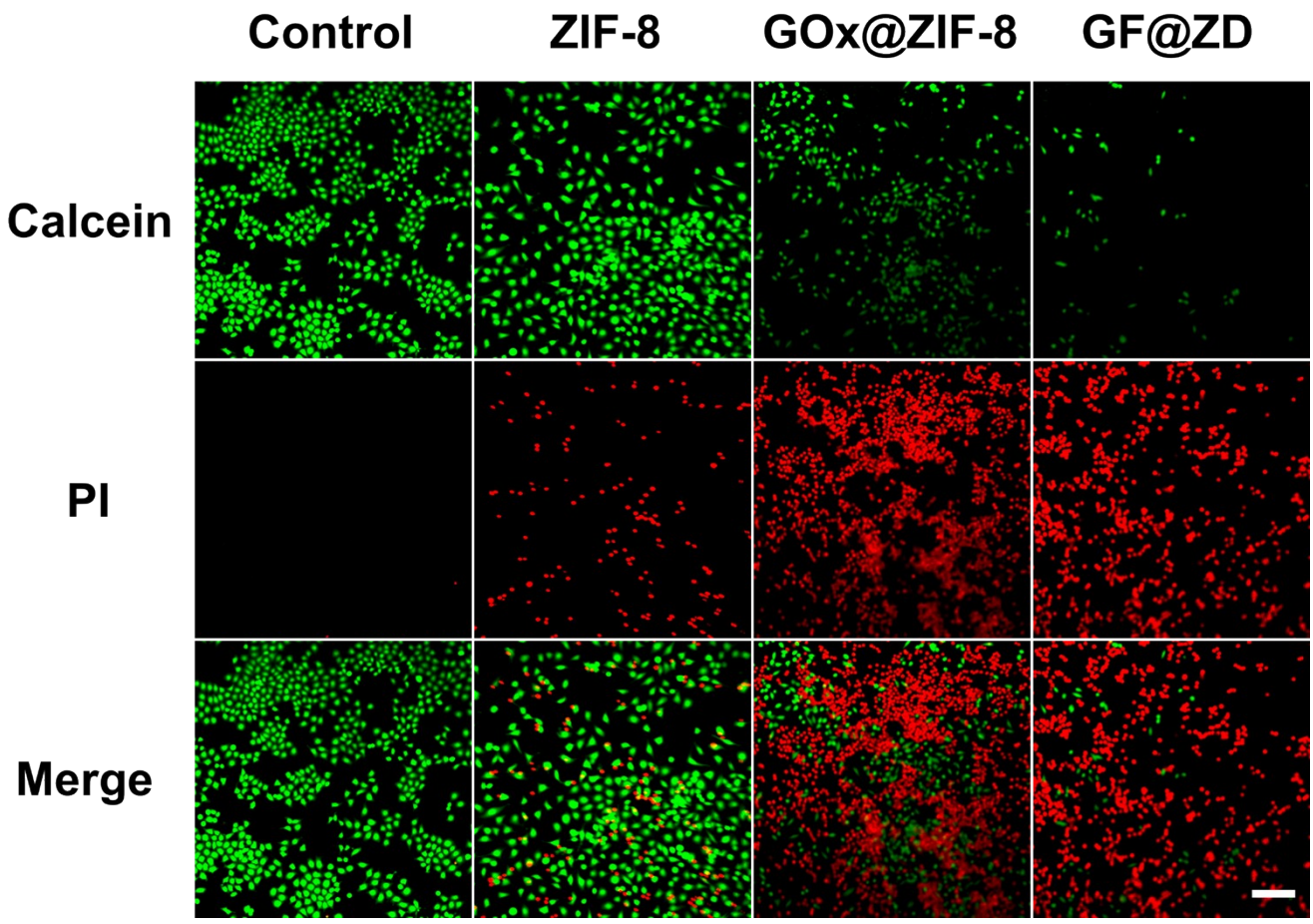

Fig. S21 Confocal fluorescence bioimaging of calcein-AM/PI co-stained MCF-7 cells after incubation with ZIF-8 (90 μg/mL), GOx@ZIF-8 (90 μg/mL) and GF@ZD (90 μg/mL); λ_{ex} = 488 nm, λ_{em} = 500 ~ 540; λ_{ex} = 561 nm; λ_{em} = 570 ~ 670nm; the scale bar is 200 μm.

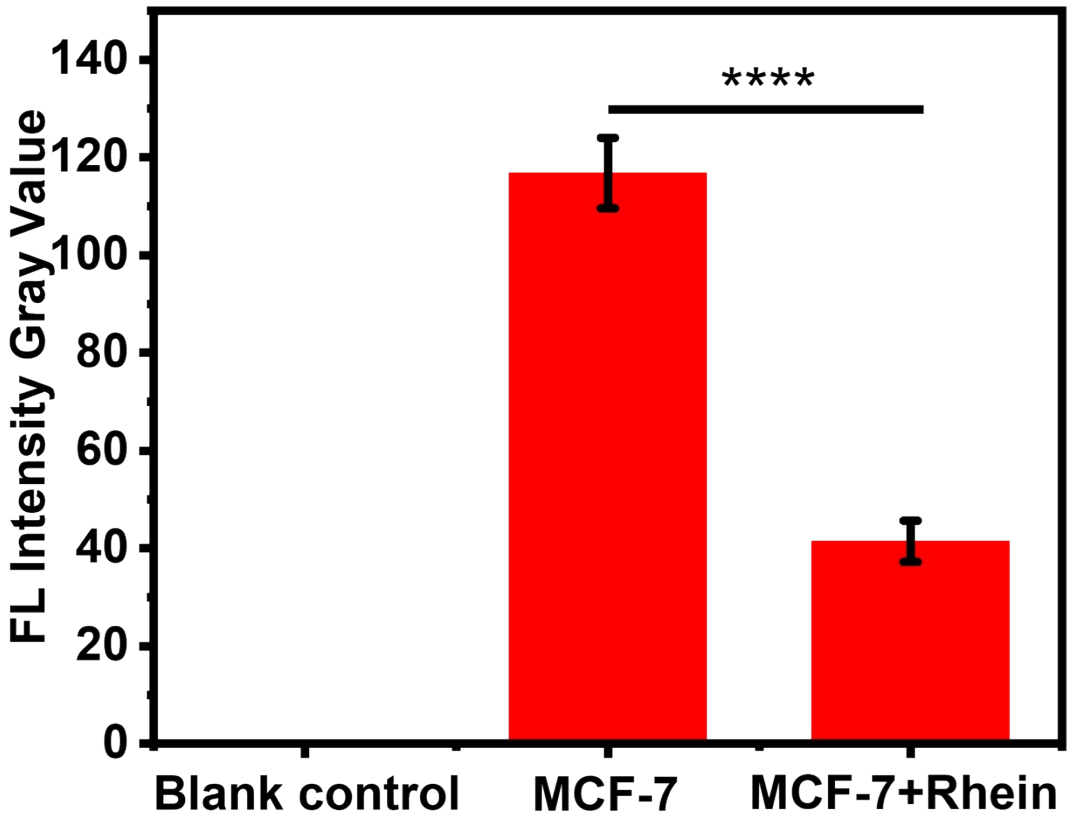

Fig. S22 Histogram of the corresponding fluorescence intensity values in Fig. 8 with ImageJ software; data are represented as mean ± SD, n = 3. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

Table S1. All oligonucleotides used in this study.

Name	Sequence (5'-3')
S-strand	S-S-TGGCCAC/iBHQ2dT/ATrAGGG/iCy3/AGAGAT
E-stand	ATCTCTCCTCCGAGCCGGTCGAAATAGTGGCCA
m^6A-E-strand	ATCTCTCCTCCGAA^{Me}GCCGGTCGAAATAGTGGCCA

rA: ribonucleotide, A^{Me}: m^6A-modified nucleotides.