Electronic Supplementary Material (ESI) for Chemical Science.

Supporting information

Synergistically Remodulating of H^+/Ca^{2+} Gradients to Induce Mitochondrial Depolarization for Enhanced Cancer Therapy

Xiaoni Wang[1], Xiyang Ge[1], Xiaowen Guan[1], Jin Ouyang[2] and Na Na[1]*

1 Key Laboratory of Radiopharmaceuticals, Ministry of Education, College of Chemistry, Beijing Normal University, Beijing 100875, China.
2 Department of Chemistry, College of Arts and Sciences, Beijing Normal University at Zhuhai, Zhuhai City, Guangdong Province 519087, China.
E-mail: nana@bnu.edu.cn

Table of contents

Experimental Procedures

Materials

The DNA, and siRNA sequences were synthesized by Sangon Biological Co. Ltd (Shanghai, China). 3,3′,5,5′-tetramethyl-benzidine (TMB) of analytical reagent grade were obtained from Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). Glutathione reduced (GSH), hydrogen peroxide (H_2O_2, 30 wt%), ammonia solution ($NH_3 \cdot H_2O$, 25 wt%), Calcium chloride anhydrous ($CaCl_2$, AR), $FeCl_3 \cdot 6\ H_2O$, $MnCl_2 \cdot 4\ H_2O$ (99%), N, N-dimethylformamide (DNA-MF, AR) 5,5′ -dithiobis-2-(nitrobenzoicacid) (DTNB) and ethanol anhydrous were purchased from Sinopharm Chemical Reagent. Dulbecco's modified Eagle's medium (DMEM), trypsin and phosphate buffered saline (PBS, pH 7.4, basic (1×)) were acquired from Sigma-Aldrich. WB Transfer Buffer (10×), MitoTracker™ DeepRed FM, BCECF-AM intracellular ratiometric pH indicator, 2',7'-dichloro fluorescein diacetate (DCFH-DA), cytochrome C rabbit and mouse monoclonal antibody, caspase-3rabbit monoclonal antibody, protease inhibitor cocktail for general use, Annexin V-FITC/PI apoptosis detection kit, mitochondrial membrane potential assay kit, Reactive Oxygen Species Assay Kit were purchased from Solarbio Science & Technology. GSH assay kit and lactate assay kit were purchased from Beyotime (China). Calcein-AM, LysoTracker Green was purchased from Thermo Fisher Scientific Inc. (Shanghai, China). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution, DNA ladder, Hoechst 33342 staining solution and Liperfluo (LPO) were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Rhod-2 AM, MDA assay kit was purchased from Beijing Solarbio Science& Technology Co. Ultrapure water (Mill-Q, Millipore, 18.2 MΩ) was used in all experiments. All chemicals were used as received without further purification. The female BALB/c nude mice (5 weeks, 18-20 g) used for animal experiments were provided by Beijing Vital River Laboratories (China).

Instruments

The transmission electron microscopy (TEM) images were obtained with a FEI Talos F200s TEM (Thermo Fisher Scientific, USA). Ultraviolet-visible (UV-vis) spectra were acquired using a Shimadzu UV-2600 spectrophotometer (Shimadzu, Japan). The fluorescence spectra were recorded on an FS5 fluorescence spectrometer (Edinburgh, UK). The dynamic light scattering (DLS) and zeta potential data were conducted using the Nano-ZS Zetasizer ZEN3600 (Malvern, UK.). On-line monitoring was performed on an LTQ XL (Thermo Fisher Scientific, USA) instrument using a homemade ionization source of ESSI-MS. The cell viability was measured by thiazolyl blue tetrazolium bromide (MTT) using a microplate reader at 490 nm on a microplate reader (BioTek, USA). Confocal fluorescence imaging of cells was performed on a Nikon A1R-si laser confocal laser scanning microscope (CLSM) (Nikon, Japan). Flow cytometric analysis was performed on CytoFLEX (Beckman, USA). Tissue slides images were captured using a Zeiss Axiovert 20 inverted fluorescence microscope (Carl Zeiss, Germany). In vivo imaging was performed on an IVIS Lumina III system (Caliper, USA).

Methods

1. Preparations.

1.1 Synthesis of MF

The monodisperse MF nanoparticles were synthesized via the thermal decomposition method according to the literature.[1] $FeCl_3 \cdot 6H_2O$ (0.043 g, 2 mmol) and $MnCl_2$ (0.013 g, 1 mmol) were mixed in 100 mL of DI water. The solution was stirred under nitrogen for 0.5 h and 45 mL of $NH_3 \cdot H_2O$ was added dropwise at 80°C. 7.5 g of citric acid in 15 mL of water was introduced after the temperature rose to 95°C and stirred for 120 min. After cooling to room temperature, the product was precipitated upon adding 20 mL ethanol and

washed with anhydrous ethanol for twice and water for twice by centrifugation (13,525 rcf, 10 min). Amine-functionalized MF. The obtained MF were redispersed in anhydrous dimethylformamide (DNA-MF, 15 mL) under sonication, and 50 μL of APTES was added and stirred at 80°C for 12 h. The amine-functionalized MF were washed with DNA-MF, anhydrous ethanol and water (twice) and dried in a vacuum at room temperature.

1.2 Synthesis of DNA Network

A 1×TAE/Mg^{2+} buffer (40 mM Tris, 20 mM acetic acid, 12.5 mM Mg^{2+}) was used to dissolve H1 and H2 strands. An annealing procedure was run (95°C for 2 min, 80°C for 2 min, slowly dropped from 80°C to 30°C with a rate of 1°C /min) to obtain DNA hairpin structures. 8 μL of N1-DNA (10 mM) was incubated with H1-dimer and H2 (H2-ssDNA) with a final concentration of 3 μM at 30°C for different time. The total volume was 20 μL. DNA-MF were synthesized by adding extra different concentration of MF in the DNA Network mixture. The resultant products were washed with water and centrifuged at 13,525 rcf for 5 min twice and then were sonicated to redisperse the DNA-MF. Furthermore, Ca^{2+} and hyaluronate acid (HA) mix in DNA-MF (100 nM) solution after stewing at 25°C for 24 h, the Ca layer was uniformly deposited on the DNA-MF NPs.

2 Polyacrylamide gel electrophoresis (PAGE) analysis

30% acrylamide (19:1, acrylamide/bisacrylamide) solution was added in 1×TAE/Mg^{2+} buffer, 75 μL ammonium persulfate and 7.5 μL tetramethyl ethylenediamine (TEMED) were used as initiating agent and accelerator separately. Each DNA sample (5 μL) was mixed with 6 × loading buffe (1 μL) and analyzed by 3% agarose gel (weight / weight) at 80 V in 1×TAE buffer for 60 min. After electrophoresis, the gels were imaged with UV channel by Champ Gel imaging system.

3 Dynamic light scattering (DLS) analysis and Zeta potential

DNA Network, MF, DNA-MF, Ca@DNA-MF were characterized by DLS measurements on a Zetasizer Nano ZS (Malvern Instruments Ltd.) to determine the size distribution. DNA Network, MF, DNA-MF, Ca@DNA-MF were characterized by Zeta potential measurements on a Zetasizer Nano ZS (Malvern Instruments Ltd.) to verify the potential.

4. Measurement of metal ions released from Ca@DNA-MF

Ca@DNA-MF was added to 900 μL of HEPES (4-hydroxyethylpiperazine ethanesulfonic acid buffer saline) with various pH conditions (pH = 7.4, 6.5 and 5.5). The mixtures were incubated at 37°C, and then filtered by centrifugal filter tube. Then aliquots of filtrate were collected at various time points and analyzed by ICP-MS to determine the concentration of Fe, Mn and Ca.

4.1 Fenton Catalytic Performance of Ca@DNA-MF

The Fenton catalytic performance of the nanoparticles with different CA@DNA-MF was monitored through a TMB color test. Briefly, 100 μL Ca@DNA-MF suspensions (200 $\mu g \cdot mL^{-1}$) were immigrated in a solution containing different pH phosphate-buffered saline, TMB (dissolved in DMSO, 1 mg/mL, 200 μL), and H_2O_2 (30 μL, 30%). After 30 min, the mixture was subjected to the absorption spectrum analysis (λ = 650 nm) via a UV-Vis spectrometer.

The $Ti(SO_4)_2$-based colorimetric method was employed to further evaluate Ca@DNA-MF-activated decomposition of H_2O_2 into ·OH. CA@DNA-MF solution was shaken at 37°C for 30 min at different pH. After centrifugation, 500 μL of supernatant was mixed with 500 μL of $Ti(SO_4)_2$ solution (obtained from 1.33 mL of 24% $Ti(SO_4)_2$ plus 8.33 mL of H_2SO_4 in 50 mL of DI water) for 10 min. Then, the absorbance at 412 nm was detected.

4.2 GSH depletion

Different quantities of DNA Network, DNA-MF were dispersed in 6 mL GSH solution (1.5 mM, pH=7.4) at 37°C. 0.5 mL supernatant was collected from the solution after centrifugation at different time (0, 0.5, 1, 1.5, 2, 2.5 and 3 h). Subsequently, 2.5 mL PBS and 50 μL 5,5'-Dithiobis-(2-nitrobenzoic acid) (10 mM, DTNB was used as the indicator of GSH) were added into the supernatant. The total amount of GSH was determined quantitatively according to the UV-vis absorbance standard curve of GSH at 407 nm.

4.3 ESR measurement

PBS solution (pH = 6.5) containing DMPO and H_2O_2 (100 μM) was added with Ca@DNA-MF (200 μg mL^{-1}). After 5 min of reaction, an aliquot of the solution was transferred to a fresh quartz tube for detecting the ·OH signal. The $O_2^{\cdot-}$ was detected by EPR spectroscopy using BMPO as the captor. PBS solution (pH = 6.5) containing BMPO and H_2O_2 (100 μM) was added with Ca@DNA-MF (200 μg mL^{-1}). Ca@DNA-MF were added into BMPO (0.5 mL) solution, after 5 min of reaction, an aliquot of the solution was transferred to a fresh quartz tube for detecting the $O_2^{\cdot-}$ signal.

Michalis-Menten kinetics

Different concentrations of H_2O_2 (2.5, 5, 20, and 100 μM) were added into PBS solution (pH = 6.5) containing Ca@DNA-MF and TMB (0.8 mg mL^{-1}). The absorbance variations of the solutions at 652 nm were measured with a microplate reader (SpectraMax M2) in a kinetic mode. Then, the absorption values can be converted into the oxTMB/generated ·OH concentration via Beer-Lambert law:

$$A = \varepsilon bc \quad (1)$$

where A is the absorption value (λ = 652 nm), ε is a constant (39,000 $M^{-1}cm^{-1}$) for the molar absorption coefficient of oxidized TMB, b is the optical distance,

and c is the oxidized TMB concentration. The Michalis-Menten kinetic curve of MSFP was obtained by plotting the initial velocities of reaction against H_2O_2 concentrations according to the equation (2):

$$V_0 = \frac{V_{max} \times [H_2O_2]}{K_M + [H_2O_2]} \quad (2)$$

where v_0 is the initial velocity of the reaction, V_{max} is the maximum velocity of the reaction, $[H_2O_2]$ is the H_2O_2 concentration in the solution, KM is the Michalis-Menten constant. Then, the values of KM and Vmax were calculated according to Lineweaver-Burk plotting (3)

$$\frac{1}{V_0} = \frac{K_M}{V_{max}} \times \frac{1}{[H_2O_2]} + \frac{1}{V_{max}} \quad (3)$$

4.4 The monitoring of the system by ambient mass spectrometry

For online monitoring of the GSH consumption, electrosonic spray ionization mass spectrometry (ESSI-MS) was constructed. As shown in Fig. S1, a quartz capillary (i.d. 0.2 mm, o.d. 0.32 mm) was inserted into the GSH and Ca@DNA-MF solution, which was surrounded by a coaxial outer quartz tube (i.d. 0.86 mm, o.d. 1.5 mm) with N_2. The sample was extracted from the solution and ionized under a high voltage of ~3 kV. The obtained ions were subsequently introduced into the MS inlet for the detections under self-pumping of high-velocity N_2.

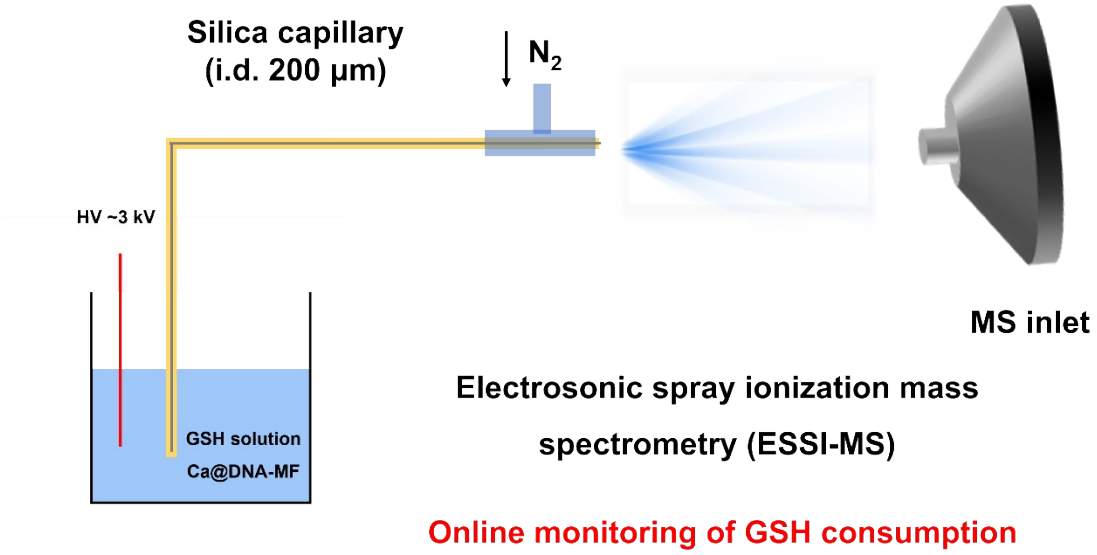

Fig. S1 Schematic diagram of ESSI-MS device for the direct extraction and ionization of samples without sample pre-treatment.

5 Cell experiments

5.1 Cell culture and MTT Assess

Cells (HeLa, 4T1, A549, HepG-2, and HEK-293T cells) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10.0% fetal bovine serum (FBS) and 1.0% penicillin/streptomycin. Cells were cultured in 5% CO_2 at 37°C.

The cytotoxicity was assessed with a standard MTT assay. 100 μL 4T1 cells (5000 cells / well) were seeded to 96-well microplates and cultured for 24 h. Then the medium was removed and 100 μL new medium containing PBS, DNA Network, MF, DNA-MF, Ca@DNA-MF (20 μg/mL) was added to each well. After incubation for 24 h, 20 μL of MTT solution (5 mg / mL) was added into each well and incubated at 37ºC for another 4 h. Finally, the supernatants were carefully removed and then 100 μL of DMSO was added to each well to dissolve the formazan crystals. Finally, the absorbance at 490 nm was measured and recorded using a microplate reader.

4T1 cells (5000 cells / well) and HEK-293T cells (5000 cells / well) were seeded to 96-well microplates and cultured for 24 h. Then, the medium was

removed and fresh medium containing Ca@DNA-MF (0-200 μg/mL) was added for further 24 h. The results were acquired using a microplate reader at 490 nm. Finally, the supernatants were carefully removed and then 100 μL DMSO was added to each well to dissolve the formazan crystals. Finally, the absorbance at 490 nm was measured and recorded using a microplate reader.

In addition, the in vitro antitumor properties were examined by MTT assay using 4T1 cells. Briefly, 4T1 cells (5000 per well) were seeded into a 96-well plate for 24 h. Then, the medium was removed and the fresh medium containing Ca@DNA-MF (0-200 μg/mL) was added for incubating for 24 h. The results were acquired using a microplate reader at 490 nm. To simulate acidic tumor microenvironment, the pH of cell culture was set at 6.5 by the addition of HCl solution (1 M, 15 μL) into the medium (980 μL).[2] Subsequently, Ca@DNA-MF was added for the incubation.

5.2 Confocal Fluorescence Imaging

To investigate the cellular uptake efficiency of Ca@DNA-MF, 4T1 cells that seeded in 35-mm glass-bottom cell culture dishes (2×10^5 cells per well) were exposed to TAMRA-Ca@DNA-MF (100 μM) and TAMRA-DNA-MF for different times (0, 4, 8 ,12 and 24 h). Then the cells were removed and washed with PBS for three times, the cells were digested by trypsin-EDTA solution with 0.25% phenol red and resuspended by PBS following by analysis with a flow cytometer (FACSAria III, BD, USA). To investigate the cellular uptake of CA@DNA-MF by 4T1 cells, cells were seeded into confocal dishes (2×10^5 cells per well) and incubated with TAMRA-Ca@DNA-MF (100 μM) for different times (4 h, 8h). Then the cells were stained by Lyso-tracker Green for 10 min and then washed by PBS for three times. Subsequently, the cells were stained by Hoechst 33342 (5 μg/mL) for another 10 min. Finally, fluorescent images were performed with a Nikon confocal laser scanning microscope, and the images were acquired using 60× objective lens. The Pearson correlation coefficient was calculated by

Image J.

5.3 In *Vivo* pH-Triggered siMCT4 Release Behavior

The pH triggered siMCT4 release from Ca@DNA-MF was investigate in different pH (5.5 6.0 6.5 6.8 7.4) environment for 24 h. Then, the fluorescence intensity of released siMCT4 was measured with an excitation of 560 nm. Evaluation of intracellular pH changes: To assess the intracellular pH changes in 4T1 cells after different treatment, 4T1 cells (≈2 × 10^5) were seeded into the confocal dish and subjected to different treatments when cell confluence reached 70%. After incubation for 12 h, the cells were washed 3 times with PBS before imaging.

5.4 Tumor Sphere Culture and Observation

The 4T1 multicellular spheroids (MCSs) were established by a liquid overlay method. Briefly, a 6-well spheroid microplate was coated with 1.5% agarose gel to prevent cell adhesion. Then, the cell suspensions (9 × 10^4 cells / 200 μL per well) were transferred into each well, gently agitated for 5 min, and maintained at 37°C for 7 days. The uniform and complete spheroids were used for the penetration studies. The nanomaterials were incubated with the tumor spheroids at 37°C for 24 h. Then, the medium was removed and the spheroids were gently washed with PBS three times. The fluorescent signal of TAMRA in spheroids was observed using the Z-stack images of the confocal microscope.

5.5 Live Cell Confocal Imaging with BCECF-AM and Mito-SypHer Transfection

To determine the intracellular pH changes, cells were pre-seeded in cell culture dishes for 24 h, and then incubated with PBS, DNA Network, MF, DNA-MF, Ca@DNA-MF for 24 h. Next, those cells were stained with BCECF-AM (2.5 μM)

for 30 min. After washing three times with PBS, the intracellular fluorescent emission of BCECF-AM was observed by Nikon A1 plus confocal laser scanning microscope (λ_{Ex} = 488 nm, λ_{Em} = 500-550 nm). The cells were loaded with PBS, DNA Network, MF, DNA-MF, Ca@DNA-MF for 24 h. Hank's Balanced Salt Solution (HBSS) was adopted to maintain a physiological pH for cells and the fluorescence of Mito-SypHer was monitored. Subsequently, the cells were stained by Hoechst 33342 (5 μg/mL) for another 10 min. Mito-SypHer was alternately excited at 405 nm and 488 nm. Fluorescence ratios (I_{488}/I_{405}) were calculated in Olympus FV31S software and analyzed in Excel (Microsoft).

5.6 Intracellular Ca^{2+} ions examination

4T1 cells were seeded into 35-mm glass-bottom cell culture dishes (2×10^5 cells per well) and respectively incubated with PBS, DNA Network, MF, DNA-MF and Ca@DNA-MF for different time (0, 4, 8, 12 and 24 h). Fluo-4 AM was a cell-permeable Ca^{2+} indicator, which reacted with the esterase within the cell to generate non-fluorescent Fluo-4. The presence of Ca^{2+} endowed Fluo-4 with strong fluorescence. Then, the cells were washed with PBS several times and stained with Hoechst 33342 for 20 min. The cells were washed with PBS several times to remove the redundant dye. Afterward, Rhod-2AM (Ca^{2+} probe) solution was added and incubated for 20 min. After removing the medium, the cells were washed thrice with PBS. Finally, the fluorescence images were recorded using inverted microscopy.

5.7 Mitochondrial Membrane Potential analysis

4T1 cells were seeded in 35-mm glass-bottom cell culture dishes (2×10^5 cells per well) and incubated overnight at 37°C and in 5% CO_2 environment. Then, the cells were incubated respectively with PBS, DNA Network, MF, DNA-MF and Ca@DNA-MF for 24 h, and then incubated with JC-1 containing DMEM-H solution for 20 min. Subsequently, the cells were stained by Hoechst 33342 (5

μg/mL) for another 10 min using a standard procedure. Finally, the samples were imaged by CLSM.

5.8 Detection of Mitochondrial Distribution

To studied the mitochondrial distribution, 4T1 cells were seeded in 35-mm glass-bottom cell culture dishes at a density of 2.0 × 10^5 cells per well and cultured for 24 h. The medium was then replaced with 1.0 mL of fresh medium with of PBS, DNA Network, MF, DNA-MF and Ca@DNA-MF for 24 h. After washing with PBS twice, the cells were stained with MitoTracker™ Deep Red FM (200 nM) for 20 min. Subsequently, the cells were stained by Hoechst 33342 (5 μg/mL) for another 20 min using a standard procedure measured by CLSM.

5.9 Investigating mPTP opening

To studied the mPTP, 4T1 cells were seeded in 35-mm glass-bottom cell culture dishes at a density of 2.0 × 10^5 cells per well cultured for 24 h. The medium was then replaced with 1.0 mL of fresh medium and treated with PBS, DNA Network, MF, DNA-MF and Ca@DNA-MF for 24 h. After washing with PBS, cells or sections were incubated with the corresponding working solution (Calcein-AM loading/ $CoCl_2$) (200 nM) at 37°C for 20 min. Subsequently, the cells were stained by Hoechst 33342 (5 μg/mL) for another 20 min and detected by CLSM.[3]

5.10 Detection of intracellular adenosine triphosphate (ATP)

4T1 cells were seeded in 35-mm glass-bottom cell culture dishes at a density of 50000 cells per well and then cultured for 24 h. Subsequently, the cells were incubated with PBS, DNA Network, MF, DNA-MF and Ca@DNA-MF at the same concentration of 200 μM for 24 h, respectively. After washing with PBS

for three times, the cells were lysed in lysis buffer at 4°C, and the lysis was collected and used for measurement of intracellular ATP level with testing kits.

5.11 Immunofluorescence Analysis:

To evaluate the changes of intracellular total Cytochrome C, 4T1 cells were seeded in 35-mm glass-bottom cell culture dishes at a density of 2.0×10^5 cells per well and cultured with PBS, DNA Network, MF, DNA-MF and Ca@DNA-MF for 24 h. After washing with PBS twice, cells were fixed with 4% (W/V) PBS-buffered paraformaldehyde for 20 min. After washing with PBS twice, cells were treated with 0.1% (V/V) Triton X-100 for 10 min and then washed with PBS. Then cells were incubated with Cytochrome C rabbit monoclonal antibody overnight at 4°C. After washing with PBS twice, cells were incubated with Alexa Fluor 647-labeled goat anti-rabbit IgG (H+L) for 40 min at 37°C. After washing with PBS, cells were stained with Hoechst 33342 (5 μg/mL) for 10 min. Finally, the fluorescence intensity was recorded by CLSM.

To test the changes of intracellular cleaved Caspase-3, 4T1 cells were seeded in 35-mm glass-bottom cell culture dishes at a density of 2.0×10^5 cells per well and treated with PBS, DNA Network, MF, DNA-MF and Ca@DNA-MF for 24 h. After washing with PBS twice, cells were fixed with 4% (W/V) PBS-buffered paraformaldehyde for 20 min. After washing with PBS twice, cells were treated with 0.1% (V/V) Triton X-100 for 10 min and then washed with PBS. Cells were treated with goat serum for 20 min. Then the cells were incubated with active pro-caspase-3 rabbit monoclonal antibody overnight at 4°C. After washing with PBS twice, cells were incubated with Alexa Fluor 647-labeled goat anti-rabbit IgG (H+L) for 40 min at 37°C. After washing with PBS, cells were stained with Hoechst 33342 (5 μg/mL) for 10 min. Finally, the fluorescence intensity was observed by CLSM.

5.12 Intracellular ROS Detection

Reactive oxygen species (ROS) generation was monitored by staining cells with DCFH-DA (Cell Biolabs inc. San Diego, CA). 4T1 (1.8×10^5) cells were seeded in 6-well plates. After incubation for 24 h, the cells were loaded with DNA Network, MF, DNA-MF, Ca@DNA-MF for 24 h. and further incubated with 1 mM DCFH-DA at 37°C for 30 min. ROS signals were then analyzed using flow cytometry.

5.13 Intracellular ·OH generation

4T1 cells were seeded into 35-mm glass-bottom cell culture dishes (2×10^5 cells per well) and respectively incubated with DNA Network, MF, DNA-MF, Ca@DNA-MF for 24 h. The cells were washed gently by PBS twice and incubated with coumarin-3-carboxylic acid (10 μM) for 20 min. Cells were stained with Hoechst 33342 (5 μg/mL) for 10 min. After washing three times with serum-free medium, the ·OH signal of the cells was recorded with a Nikon confocal laser scanning microscope.

5.14 Measurement of Intracellular LPO and AO

To assess the intracellular LPO production, 4T1 cells pre-seeded in optical cultured dishes were treated with PBS, DNA Network, MF, DNA-MF and Ca@DNA-MF (5 μg/mL) for 24 h. After being washed with PBS for 3 times, those cells were stained with 10 μM of C11-BODIPY and Hoechst (1 μg/mL) for 35 min, respectively. Finally, the fluorescent emission of LPO (E_x = 488 nm, E_m = 530 nm) from those cells was detected using CLSM to test intracellular LPO. Cells were stained with Hoechst 33342 (5 μg/mL) for 10 min. In addition, the same experimental setup was used for the flow cytometry analysis.

To assess the intracellular AO production, 4T1 cells were pre-seeded in optical cultured dishes for treating with PBS, DNA Network, MF, DNA-MF and Ca@DNA-MF for 24 h. After being washed with PBS for 3 times, those cells were stained with AO (10 μM) for 35 min. Cells were stained with Hoechst

33342 (5 μg/mL) for 10 min. The fluorescent emission of AO (E_x = 488 or 561 nm) from was detected using CLSM to test intracellular AO.

5.15 Measurement of Intracellular MDA Abundance

To evaluate the inhibition effect of Ca@DNA-MF on intracellular MDA, 4T1 cells (≈1 × 10^5) were seeded in 96-well plates. When the cells confluence reached 70%, the cells were washed with PBS three times and media containing different samples were added including PBS, DNA Network, MF, DNA-MF and Ca@DNA-MF. The cells were further cultured for 24 h and the changes in intracellular MDA levels were measured d by TBA kit according enzyme labeled instrument system according to the manufacturer's protocol (Bain-marie Biotech, China).

5.16 Measurement of Intracellular GSH Abundance

To test the depletion effect of Ca@DNA-MF on intracellular GSH, 4T1 cells (≈1 × 10^5) were seeded in 96-well plates and incubated using the protocol above. When the cells confluence reached 70%, the cells were washed with PBS three times and media containing different samples were added including PBS, DNA Network, MF, DNA-MF and Ca@DNA-MF. The cells were further cultured for 24 h were measured according to the manufacturer's instructions.

5.17 Dead/live staining

4T1 cells were seeded into 35-mm glass-bottom cell culture dishes at 37°C for 24 h. After which, previous medium was replaced by fresh medium that contained with PBS, DNA Network, MF, DNA-MF and Ca@DNA-MF (200 μg/mL). After co-culture for 24 h, cells were stained with Calcein AM (1.5 μL) and PI (1.5 μL) for 20 min and then imaged using CLMS.

5.18 Cell Apoptosis Assay

4T1 Cells were seeded in 35-mm glass-bottom cell culture dishes with a density of 2 × 10^5 cells per well for 24 h and then treated with free PBS, DNA Network, MF, DNA-MF and Ca@DNA-MF (200 μg/mL) for 24 h. The supernatant and the cell monolayer were collected, washed twice with cold PBS. Then, they were stained with Alexa Fluor 488 annexin V and Propidium Iodide using an Alexa Fluor 488 annexin V/Dead cell apoptosis kit according to the manufacturer's instructions. After staining, the percentage of apoptotic cells was examined with flow cytometry.

6 Tumor-bearing mice experiments

6.1 Models

All animal experiment protocols were reviewed and approved by the Animal Care and Use Committee of Institute of Beijing Normal University and complied with all relevant ethical regulations. The female BALB/c nude mice (18-20 g, 5 weeks) were purchased from Beijing Charles River Laboratory Animal Center and raised in a specific pathogen-free grade laboratory. The 4T1 tumor-bearing mice model was established by subcutaneously inoculating about 5×10^6 cells in the right flank of BALB/c nude mice.

6.2 In vivo Imaging

When the average tumor volume reached 100-120 mm^3, the mice were intravenously injected with PBS, MF, DNA-MF and Ca@DNA-MF, at 30 nmol kg^{-1} dosages, respectively. Fluorescence imaging data were collected using the IVIS Lumina II in vivo imaging system at 6 h post-injection. The mice were euthanized and the main organs (liver, kidney, spleen, lung, and heart) of the mice were selected for ex vivo imaging by IVIS Lumina II in the vivo imaging system.

6.3 Tumor growth inhibition

For in vivo antitumor study, the mice were randomly divided into four groups (three mice per group) for the treatment to obtain the tumor volume of about 100-120 mm^3. 200 μL of therapeutic nanoagents (1 mg kg^{-1} siRNA) was injected into each nude mouse via tail vein every other day for 14 days and meanwhile tumor weight and size were monitored. Tumor size was measured by a caliper and the tumor volume was calculated according to the following formula:

tumor volume (mm^3) = length×$width^2$/2

6.4 Histology examination, Ki-67, and TUNEL assay.

Red blood cells (RBCs) were collected and purified to be resuspended in 5 mL saline. 0.2 mL of RBCs was incubated with 0.8 mL Ca@DNA-MF in saline at different concentrations for 30 min at 37°C, respectively. Ca@DNA-MF, saline (negative control) and MQ (positive control) were incubated with RBCs, respectively. After centrifugation (6010 g, 5 min), the supernatant was obtained to detect the absorbance at 570 nm with a microplate reader. The hemolysis (%) was calculated by the following equation:

$$Hemolysis = \frac{Asample - Anegative}{Apositive - Anegative} \times 100\% \quad (4)$$

where A sample is the absorbance of supernatant after incubation of blood and materials, Anegative and Apositive are the absorbance of supernatant after incubation of saline and H_2O, respectively.

At the 14^{th} day, the heart, liver, spleen, lung, kidney, and tumor from every group were excised, fixed in 4% paraformaldehyde solution. Then, the tissues were sectioned for histopathological analysis with H&E staining, TUNEL immunofluorescence assay and Ki-67 staining. For histology analysis, tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Paraffin-

embedded serial sections (5 μm) were obtained, stained with hematoxylin solution and eosin solution (H&E), Ki-67 staining to assess histological alterations by microscopy.

Apoptotic cell death in the tumor tissue was measured by TUNEL assay using an in-situ cell death detection kit according to the manufacturer's protocol. Images were acquired by fluorescence microscopy. The paraffin-embedded 5-μm-thick tumor sections were mounted on glass slides, and heated for 30 min at 60°C. Followed by incubations in a bath at room temperature, the deparaffinized slides were rehydrated by graded alcohols (100%, 95%, 85%, 75%) and washed in distilled water. The slides were treated with freshly prepared PBS for 10 min at room temperature and washed three times with distilled H_2O for 2 min. Then slides were covered by 20 μg/mL of proteinase K solution (1 mL) for 15 min at room temperature and washed with distilled H_2O three times subsequently. The excess water on the slides were blotted away carefully. Then, the slides were incubated with TdT equilibration buffer (2.5 mM Tris-HCl (pH~6.6), 0.2 M potassium cacodylate, 2.5 mM $CoCl_2$, 0.25 mg/mL bovine serum albumin (BSA) for 10 min at room temperature. After removing the TdT equilibration buffer, the slides were incubated with TdT reaction buffer for 60 min (at 37°C). The slides were carefully covered with a glass coverslip and washed three times with distilled PBS for 5 min at room temperature. Subsequently, the slides were incubated with 2% BSA solution for 30 min at room temperature. After washed with PBS and stained with Hoechst 33342, the slides were washed with PBS again, dried in air, and attached coverslips by Fluoromount-G™ Anti fluorescence quenching sealing agent. The tissue sections were examined by laser confocal fluorescence microscopy (Zeiss) at an excitation wavelength of 488 nm with emission wavelength at 510-550 nm. Hoechst 33342 was excited by a 405 nm laser with emission collected at 420-460 nm.

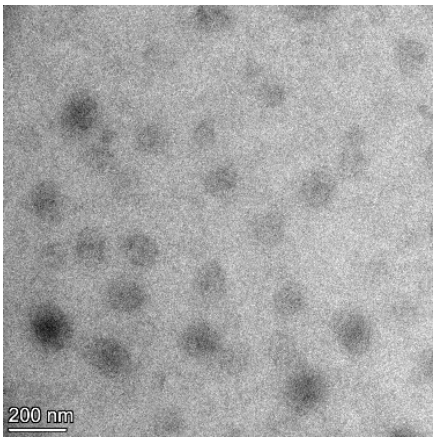

Fig. S1 TEM images of DNA Network.

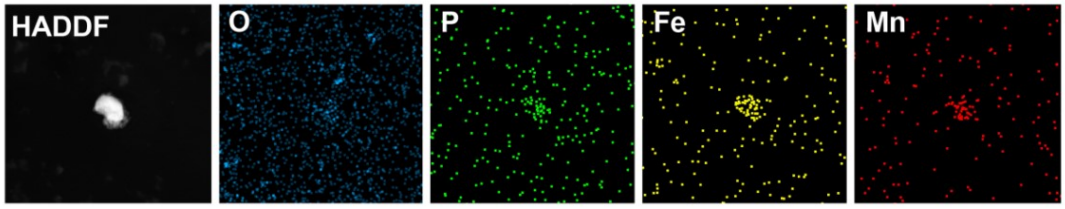

Fig. S2 HAADF-STEM image and corresponding element mapping analysis of DNA-MF.

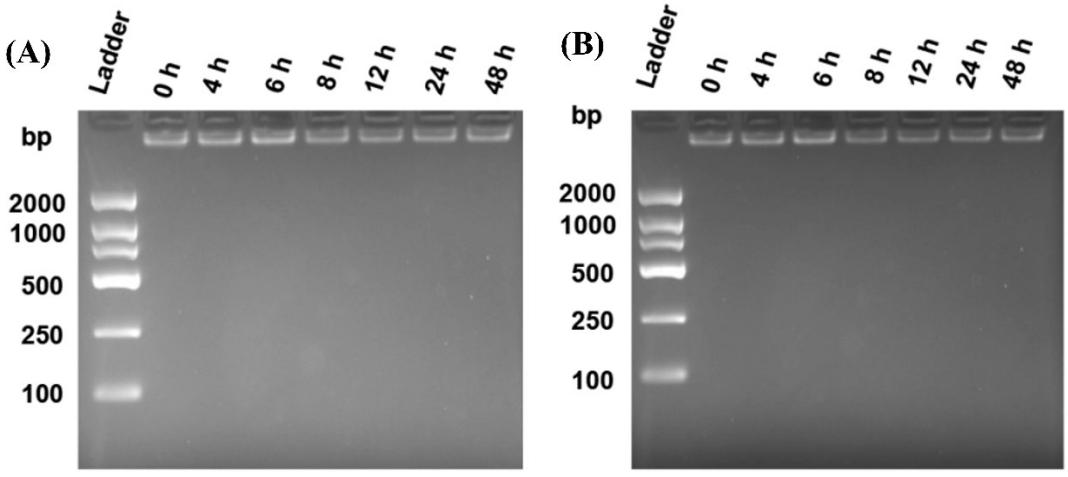

Fig. S3 Stability of nanocarriers. Agarose gel electrophoresis analysis of DNA Network (A) and Ca@DNA-MF (B) after a preassigned incubation in a culture medium (RPMI-1640) with 10% fetal bovine serum.

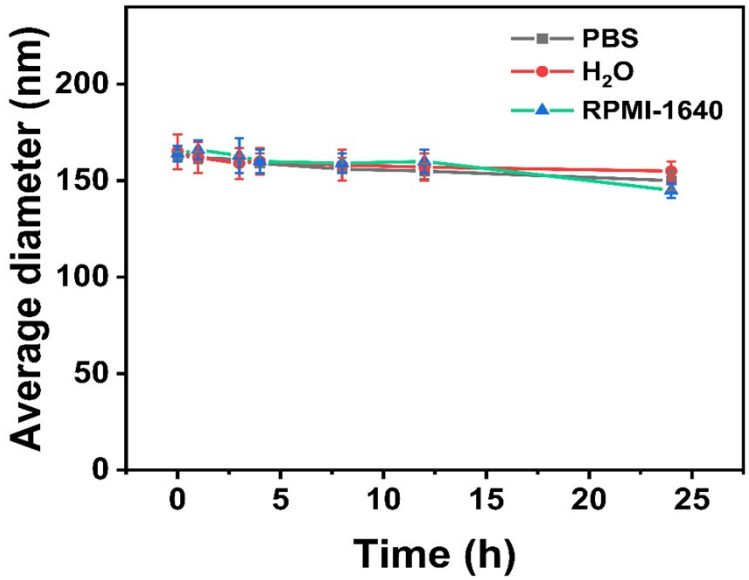

Fig. S4 Size stability (by Intensity) of Ca@DNA-MF in water, culture medium (RPMI-1640), and PBS (pH = 7.4, contain 10% FBS) over time.

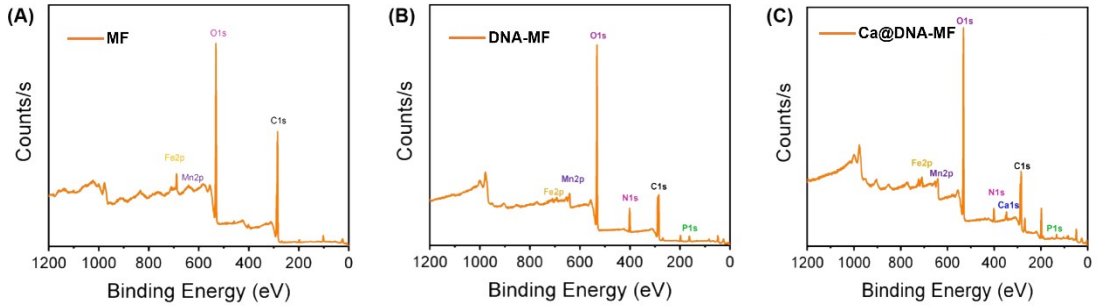

Fig. S5 High-resolution XPS spectrum of MF (A), DNA-MF (B) and Ca@DNA-MF (C).

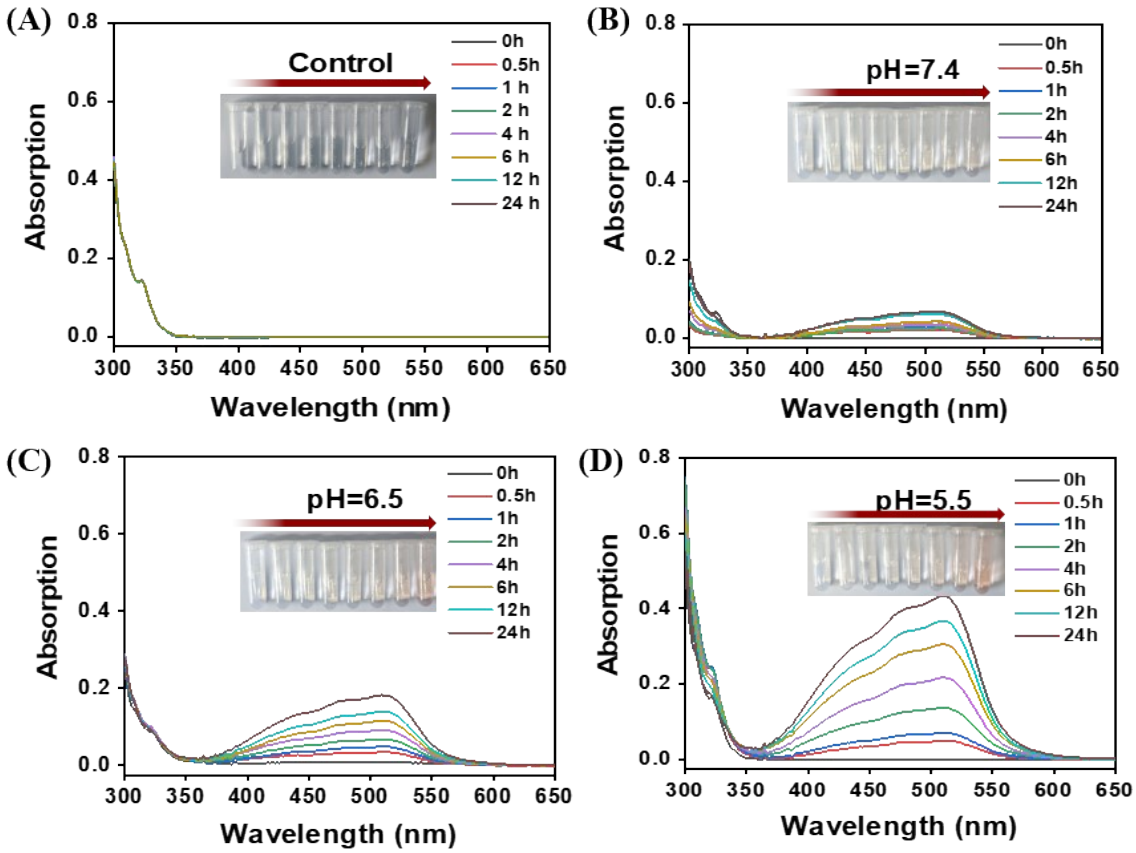

Fig. S6 The UV-vis spectra for Fe^{2+} detection using 1,10-phenanthroline as the probe when Ca@DNA-MF dispersed in different solutions. (A) Control without Ca@DNA-MF. (B) pH 7.4 buffer, (C) pH 6.5 buffer, D) pH 5.5 buffer.

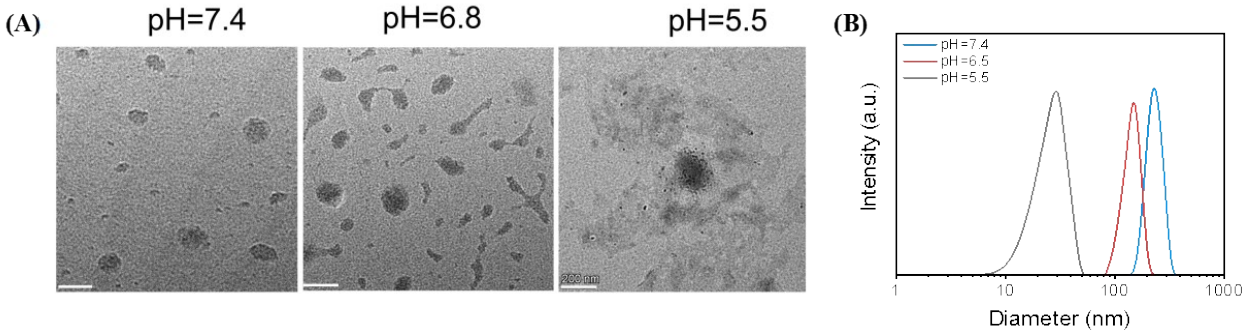

Fig. S7 TEM image (A) and DLS (B) of Ca@DNA-MF at different pH conditions.

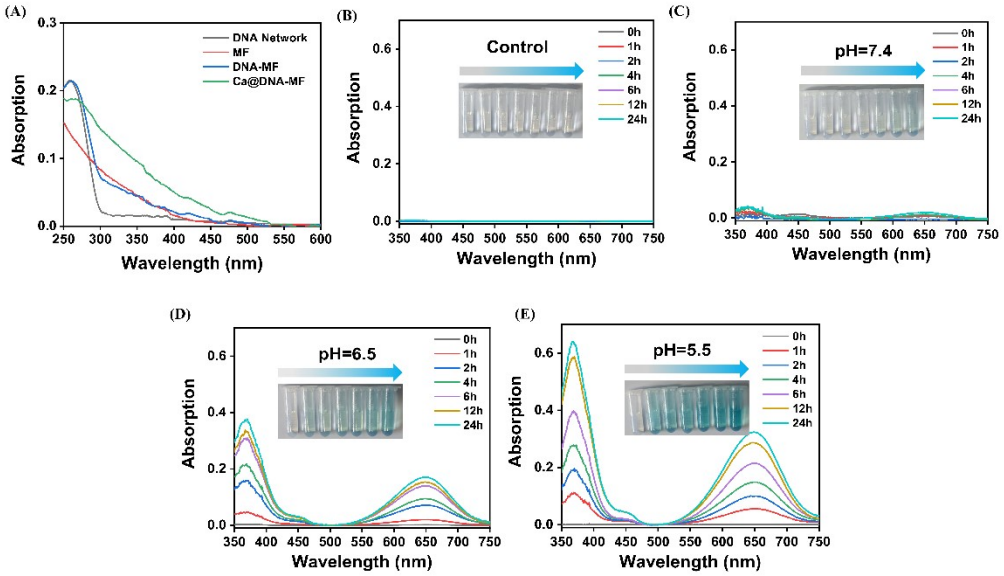

Fig. S8 (A) The UV-vis spectra of different materials (DNA Network, MF, DNA-MF, Ca@DNA-MF). (B) The control without Ca@DNA-MF NPs. The UV-vis spectra for ·OH detection using TMB as the probe when Ca@DNA-MF NPs dispersed in solutions at (C) pH 7.4, (D) pH 6.5, (E) pH 5.4. H_2O_2: 0-20 μM.

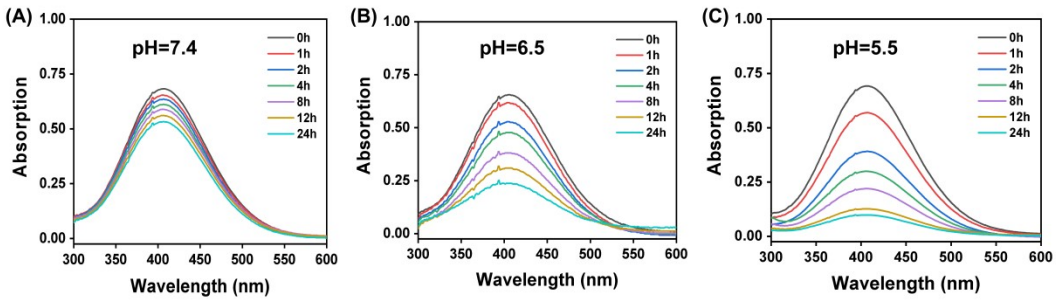

Fig. S9 Ca@DNA-MF-mediated H_2O_2 degradation, which was evaluated by titanium sulfate in different PBS (pH 7.4, pH 6.5, pH 5.5) buffer.

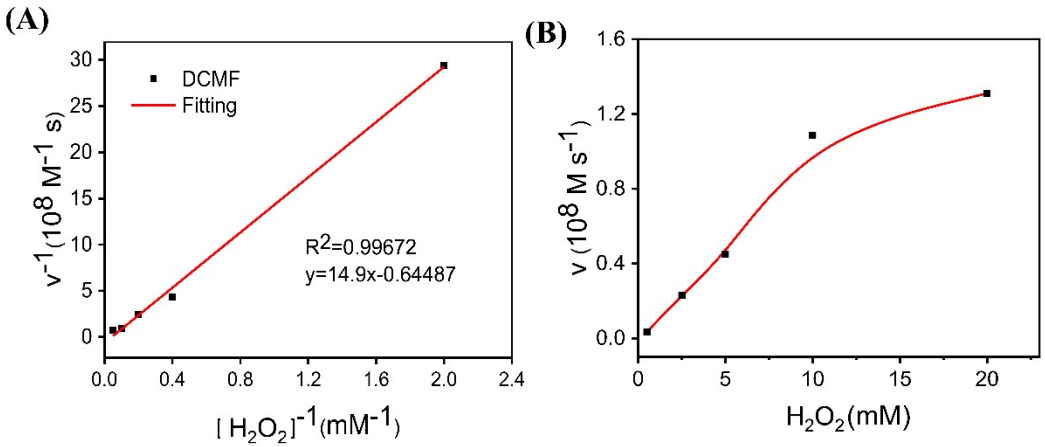

Fig. S10 Michaelis-Menten kinetics of Ca@DNA-MF generated from Fig. 2F. (B) Lineweaver–Burk plotting of Ca@DNA-MF according to (A).

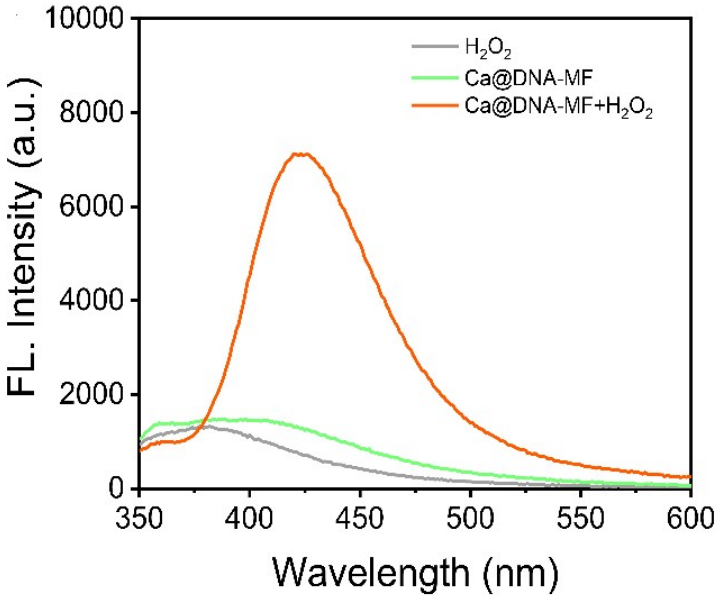

Fig. S11 Generation of ·OH, which as indicated by the enhanced fluorescence of TA.

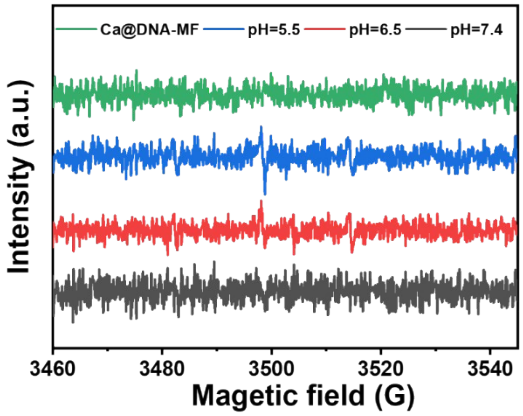

Fig. S12 EPR spectra of the $O_2^{\bullet-}$ in the Ca@DNA-MF system and the groups in the presence of air or H_2O_2 at pH 5.5, 6.5 and 7.4.

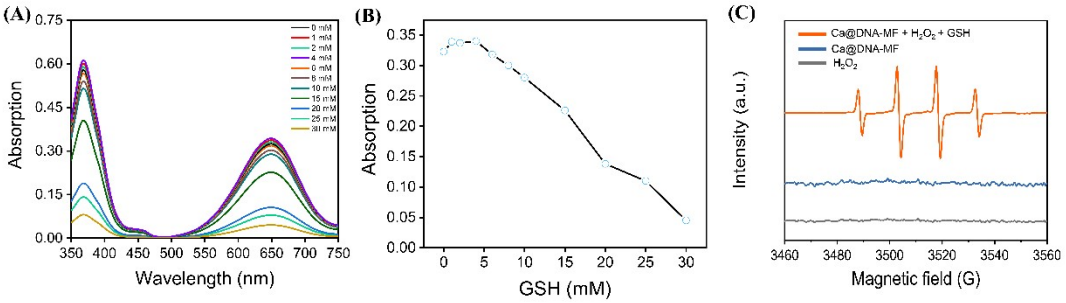

Fig. S13 (A) Ca@DNA-MF treated with H_2O_2 (20 mM) and different concentration of GSH (0-30 mM). (B) Different peak values in (A). (C) ESR spectra of Ca@DNA-MF in different solutions.

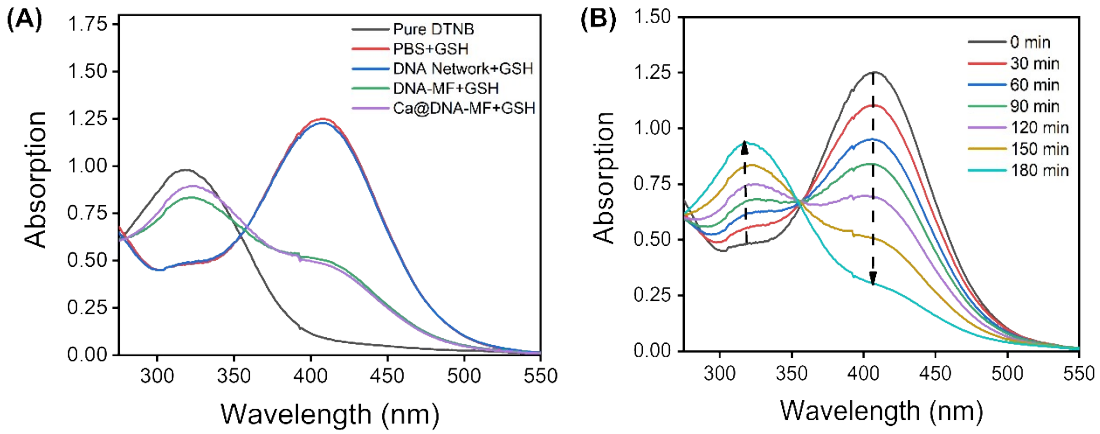

Fig. S14 (A) GSH depletion by different materials, which was measured by DTNB. (B) UV curves for evaluating GSH concentration after incubating with Ca@DNA-MF (HEPES buffers at pH 6.5) for different times.

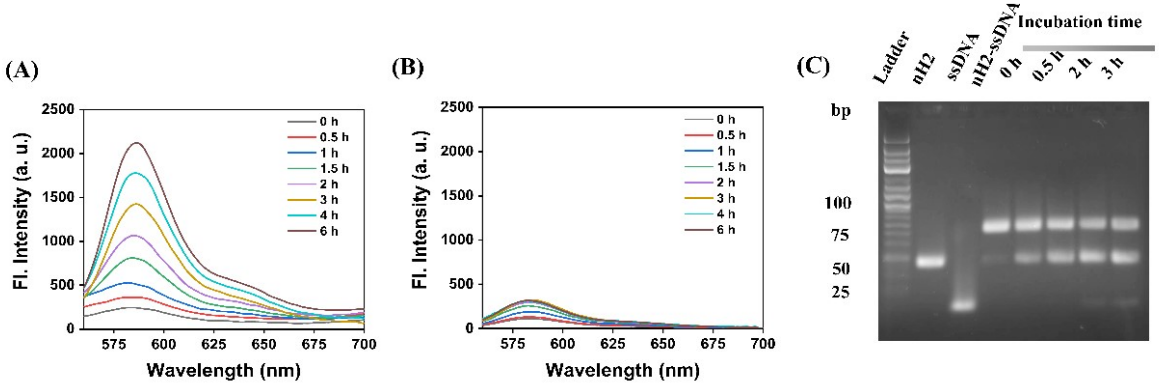

Fig. S15 FL response of ATP triggered disassembly of H2-ssDNA in which ssDNA was linked via an ATP aptamer sequence (A) and via an nATP aptamer sequence (nATP-DNA Network) (B). (C) Gel electrophoresis image of nATP-H2-ssDNA (2 μM) incubated with 10 mM ATP at 37°C for indicated time.

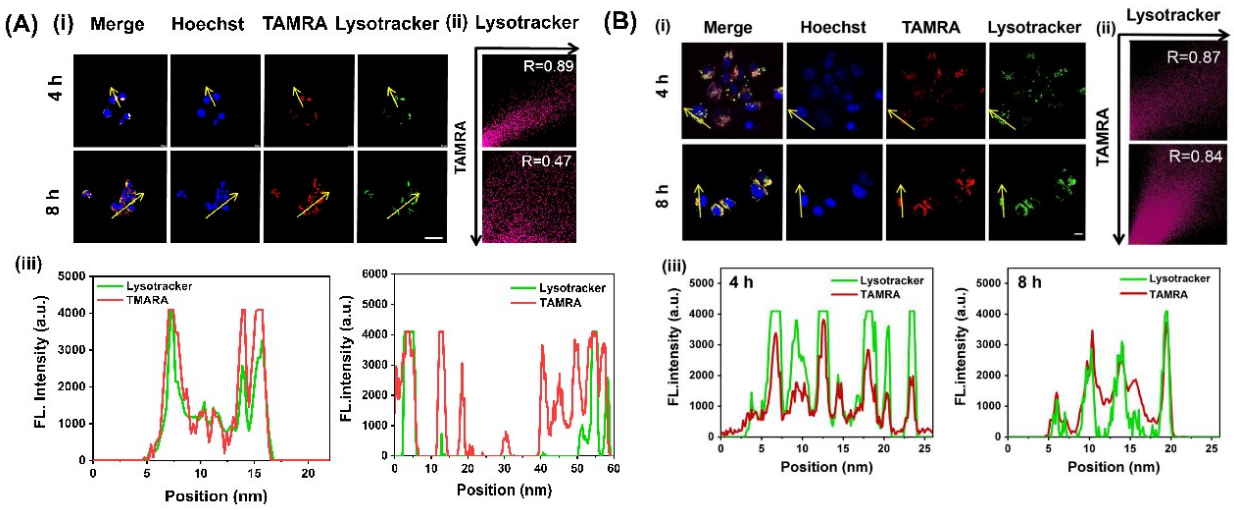

Fig. S16 Colocalization coefficient examinations. (A) Colocalization coefficient between the Lysotracker Green and Ca@DNA-MF after incubating for 4 h and 8 h (i). (ii) PCCs of Lyso-tracker-TAMRA. (iii) The intensity distribution of Lysotracker Green and TAMRA along the arrow in (i). Scale bars: 10 μm. (B) Colocalization coefficient between the Lysotracker Green and DNA Network with different incubation times (i). (ii) PCCs of Lyso-tracker-TAMRA. (iii) The intensity distribution of Lysotracker Green and TAMRA along the arrow in (i). Scale bars: 50 μm.

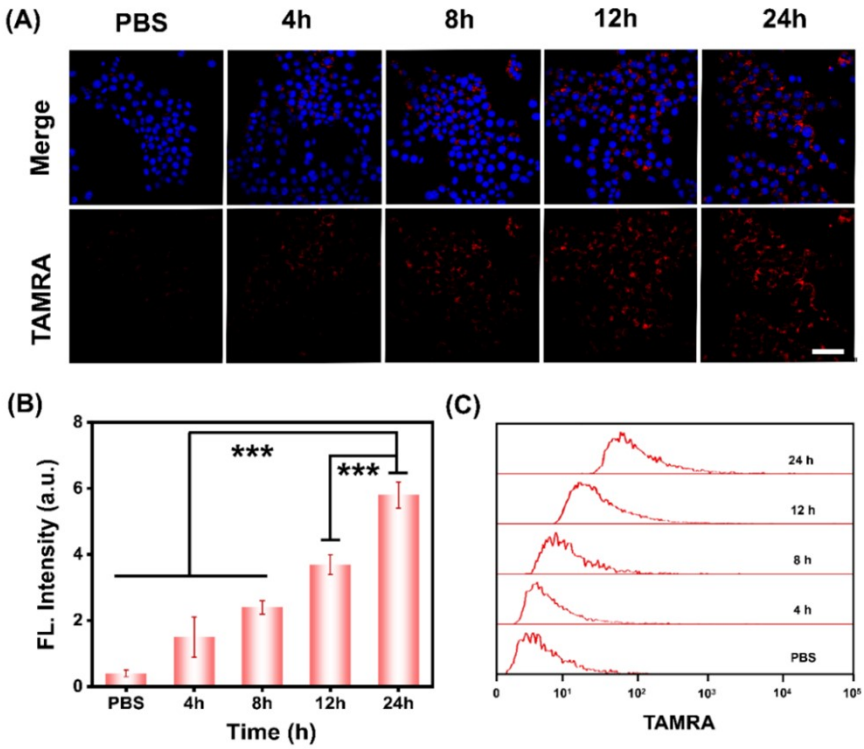

Fig. S17 (A) Confocal microscopic images after incubation of DNA-MF with 4T1 cells for different time and (B) fluorescence intensity of each group in (A). (C) Flow cytometry after incubation of DNA-MF with 4T1 cells for different time. Scale bars: 50 μm. The statistical analysis was performed in contrast to a control group (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, t-test).

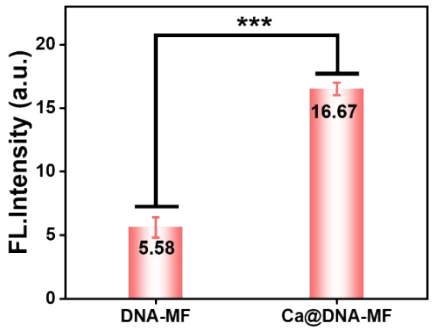

Fig. S18 Comparison of fluorescence intensity of 4T1 cells after incubating with Ca@DNA-MF (Fig. 3D-i) and DNA-MF (Fig. S17-B) for 24 h. The statistical analysis was performed in contrast to a control group (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, t-test).

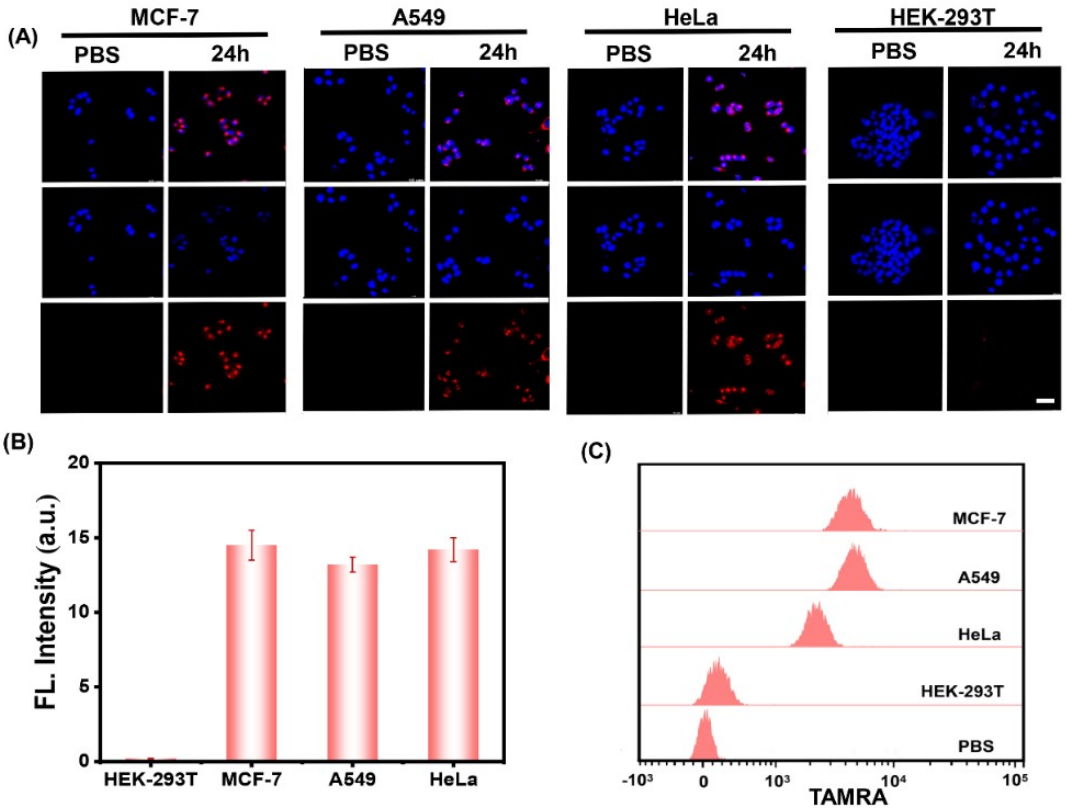

Fig. S19 (A) Confocal microscopic images of cells (MCF-7, A549, HeLa, and HEK-293T) after incubation with Ca@DNA-MF for 24 h. (B) Fluorescence intensity of each group in (A). (C) Flow cytometric fluorescence intensity of cells (MCF-7, A549, HeLa and HEK-293T) after incubation with Ca@DNA-MF for 24 h. Scale bars: 100 μm.

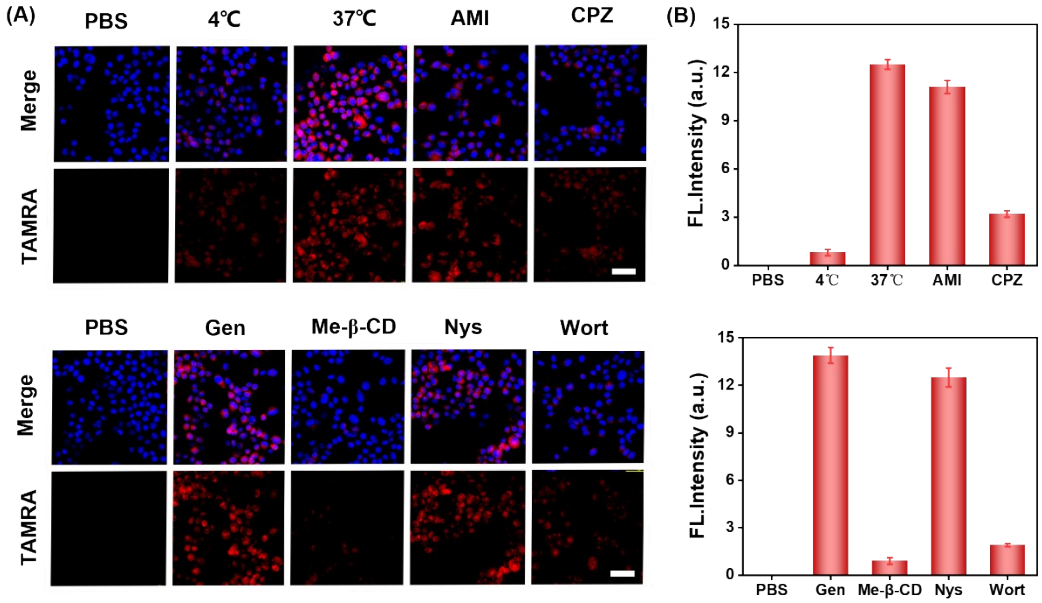

Fig. S20 Studies of the endocytosis pathway of Ca@DNA-MF. Confocal images of 4T1 cells were treated with different endocytosis inhibitor and then incubated with 100 μg/mL Ca@DNA-MF for 24 h. Endocytosis inhibitors including Amiloride (AMI, macro-pinocytosis mediated endocytosis inhibitor), Chlorpromazine hydrochloride (CPZ, clathrin-mediated endocytosis inhibitor), Genistein (Gen, pit mediated endocytosis inhibitor), Methyl-β- cyclodextrin (Me-β-CD, lipid-raft mediated endocytosis inhibitor), Nystatin (Nys, caveolin-mediated endocytosis inhibitor), and wortmannin (Wort, micropinocytosis inhibitor) were employed in the experiment. Scale bar:50 μm. (B) Fluorescence intensity of each group in (A).

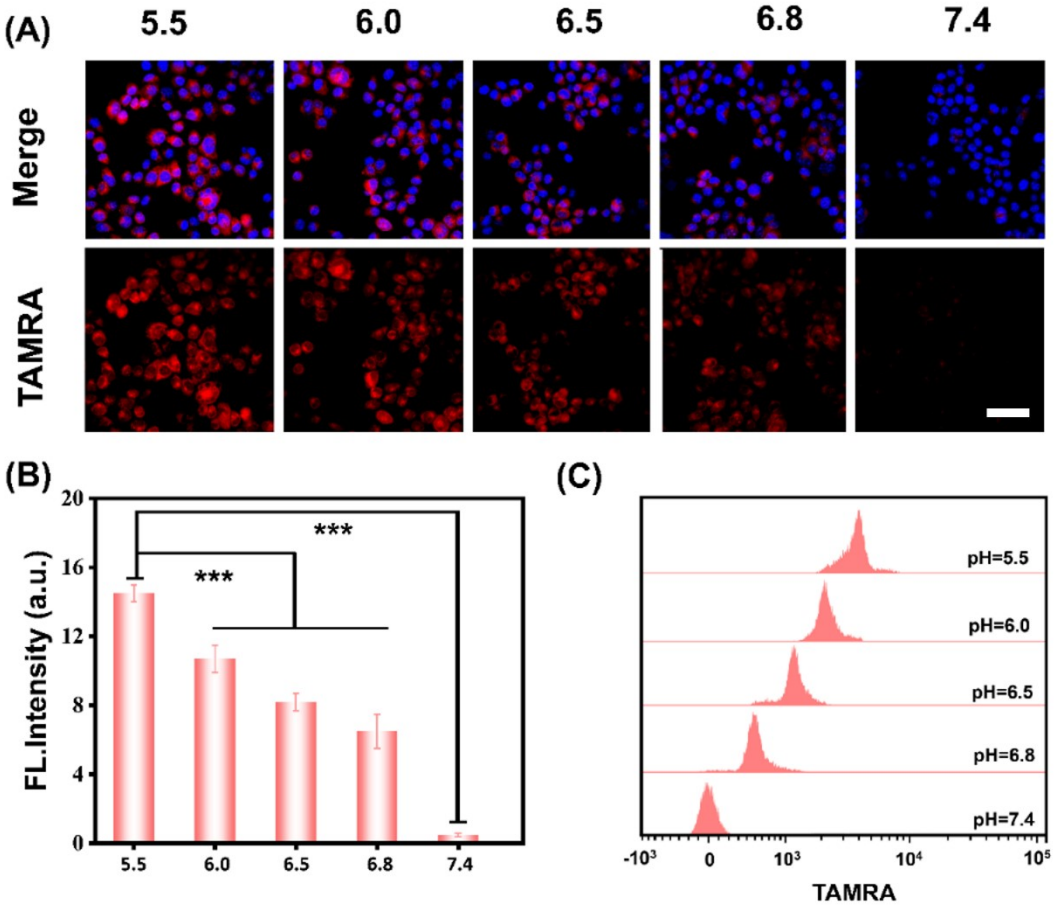

Fig. S21 (A) Confocal microscopic images after incubation of Ca@DNA-MF with 4T1 cells at different pH values for 24 h. Scale bars: 50 μm. (B) Fluorescence intensity of each group in (A). (C) Flow cytometric fluorescence intensity of 4T1 cells after incubation of Ca@DNA-MF with 4T1 cells at different pH for 24 h. The statistical analysis was performed in contrast to a control group (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, t-test).

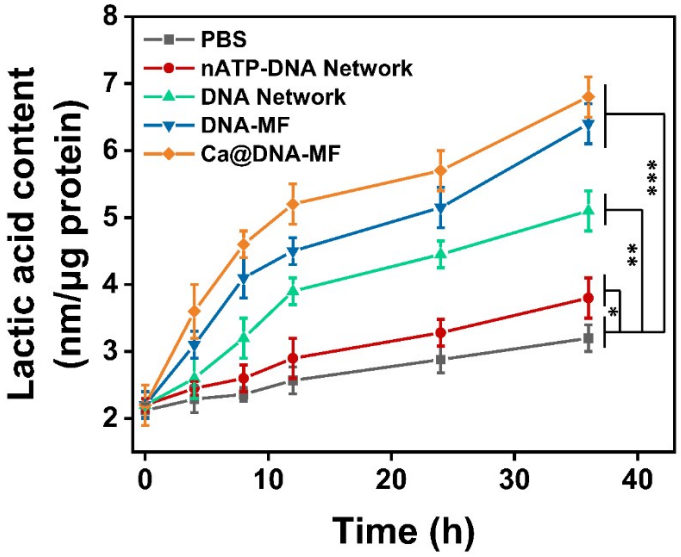

Fig. S22 Lactic acid content measurement after different treatments by Lactic acid assess kit. The statistical analysis was performed in contrast to a control group (* p<0.05, ** p<0.01, *** p<0.001, t-test).

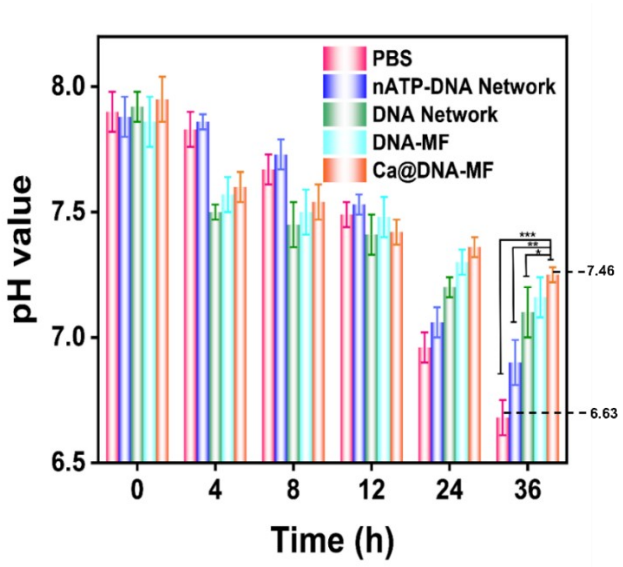

Fig. S23 The pH changes of the supernatants of 4T1 cells after incubation with PBS, nATP-DNA Network, DNA Network, DNA-MF and Ca@DNA-MF for 24 h. The statistical analysis was performed in contrast to a control group (* p<0.05, ** p<0.01, *** p<0.001, t-test).

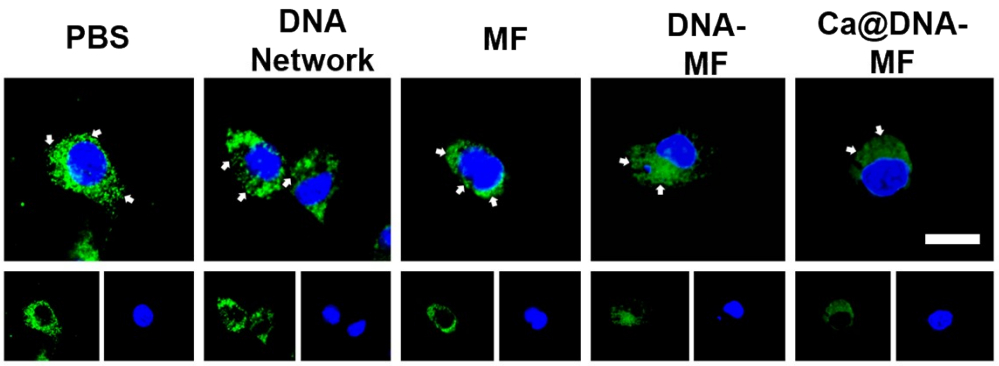

Fig. S24 Mitochondrial morphology imaging of MitoTracker Green-stained 4T1 cells after incubation with PBS, DNA Network, MF, DNA-MF and Ca@DNA-MF for 24h. Scale bar: 20 μm.

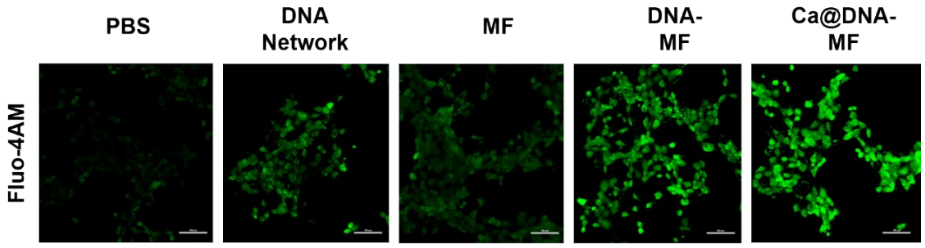

Fig. S25 Imaging of the intracellular Ca^{2+} in the cytoplasm treated PBS, DNA Network, MF, DNA-MF and Ca@DNA-MF for 24 h. Scale bars:100 μm.

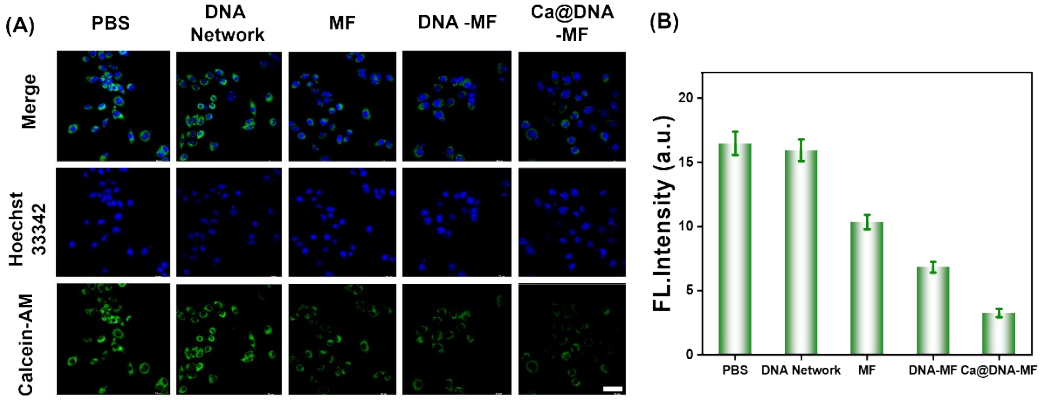

Fig. S26 The evaluation of mitochondrial permeability transition pore (mPTP) during mitochondrial depolarization of 4T1 cells by different materials. (A) FL imaging of 4T1 cells by the calcein-AM loading/$CoCl_2$ quenching strategy. (B) Fluorescence intensity of each group in (A).

After incubating with different nanomaterials, the mPTP of 4T1 cells was evaluated by

fluorescent imaging with the probe of calcein-AM whose FL signals can be quenched by $CoCl_2$ (*Adv. Healthcare Mater*. 2023, **12**, 2203106). During imaging, both cytoplasm and mitochondria can be imaged upon the distribution of calcein-AM into the cytoplasm and mitochondria. With the addition of $CoCl_2$ into the healthy cells without mitochondrial depolarization, the FL signals of calcein in the cytoplasm could be quenched. While the signal in the mitochondria would not be changed, because the mPTP with a "flickering" opening state could exclude $CoCl_2$ from the mitochondria. However, for the cells of mitochondrial depolarization, the persistent opening of mPTPs could be induced to facilitate the FL quenching upon the entering of $CoCl_2$ into mitochondria. Thus, the decrease of fluorescence can be used to evaluate the activation of mPTP. As resulted (Fig. S26), the lowest green FL signals were recorded in the group of Ca@DNA-MF, which indicated the most active mPTP opening levels upon the mitochondrial depolarization.

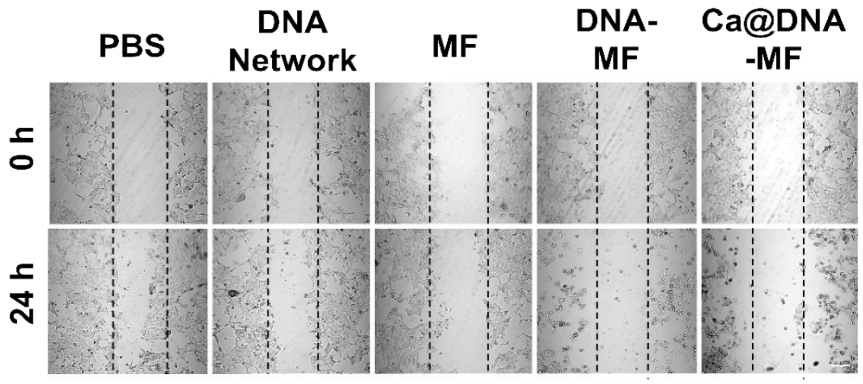

Fig. S27 Images of wound healing assays pre-treated with PBS, DNA Network, MF, DNA-MF and Ca@DNA-MF. for 24 h. Scale bars: 100 μm.

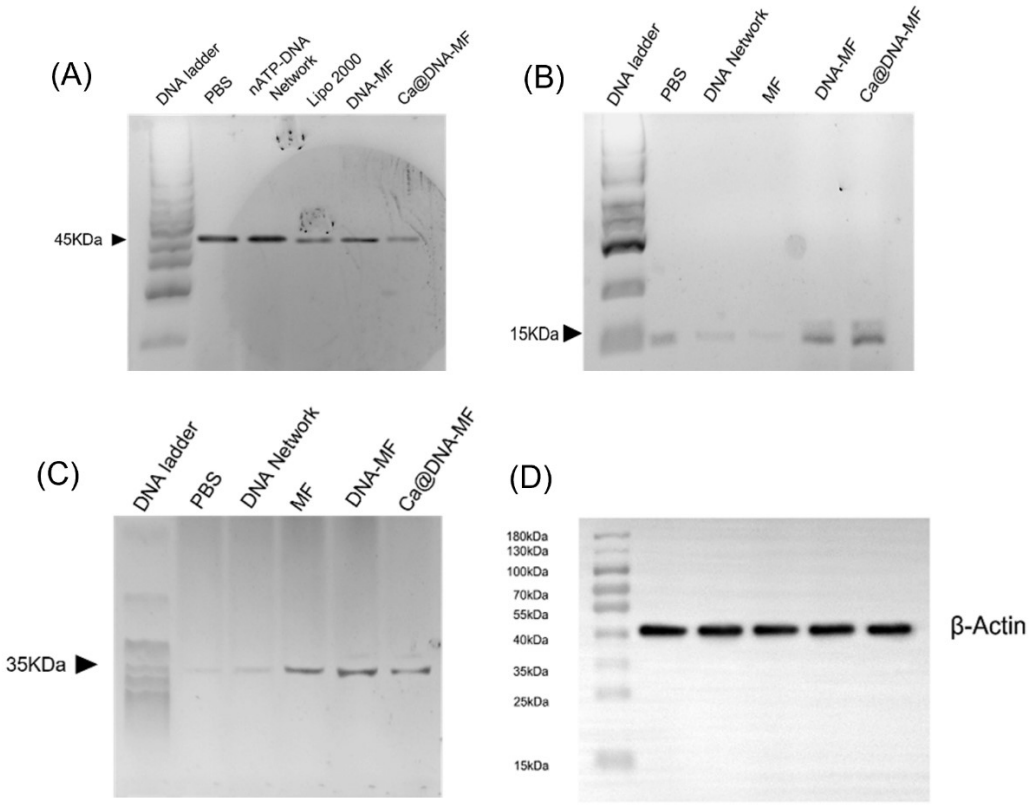

Fig. S28 Western blotting assay. (A) MCT4 inhibition. The mitochondria apoptosis indicated by contents of Cytochrome C (B) and Caspase-3 (C). Control: β-Actin (D).

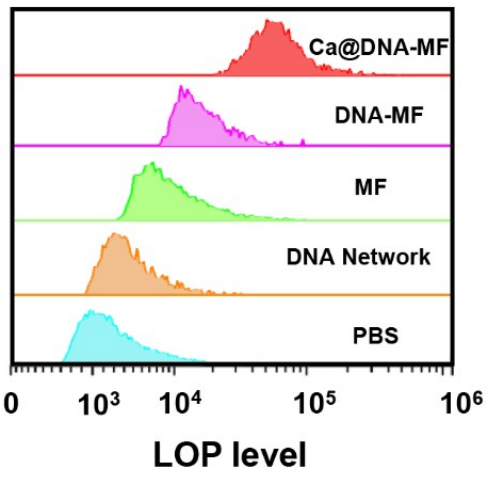

Fig. S29 Flow cytometric analysis on the intracellular LPO levels in 4T1 cell incubated with PBS, DNA Network, MF, DNA-MF and Ca@DNA-MF for 24 h.

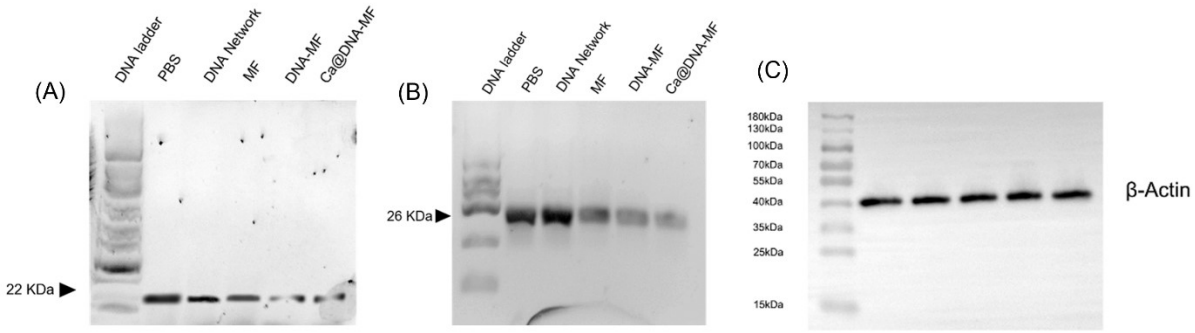

Fig. S30 Western blotting assay to indicate the therapy performance. (A) The intracellular GPX-4 proteins. (B) Bcl-2 proteins. (C) Control: β-Actin.

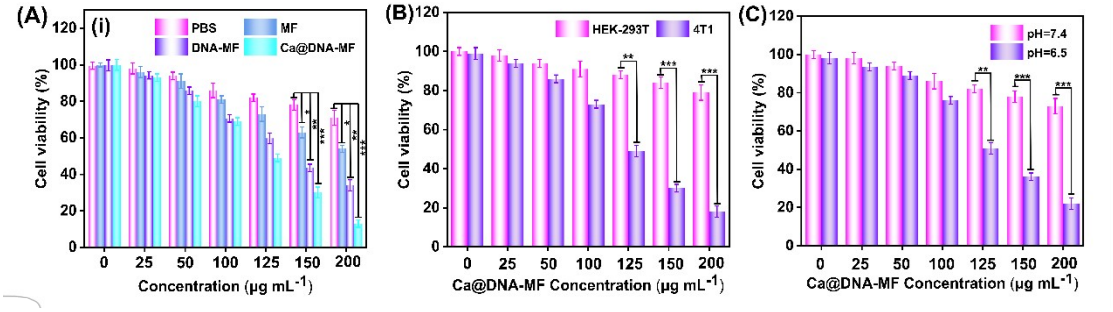

Fig. S31 MTT assay. (A) For different nanomaterials (PBS, MF, DNA-MF, Ca @DNA-MF). (B) For different concentrations of Ca@DNA-MF in different cells. (C) For 4T1 cells treated with Ca@DNA-MF at different pH. The statistical analysis was performed in contrast to a control group (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, t-test).

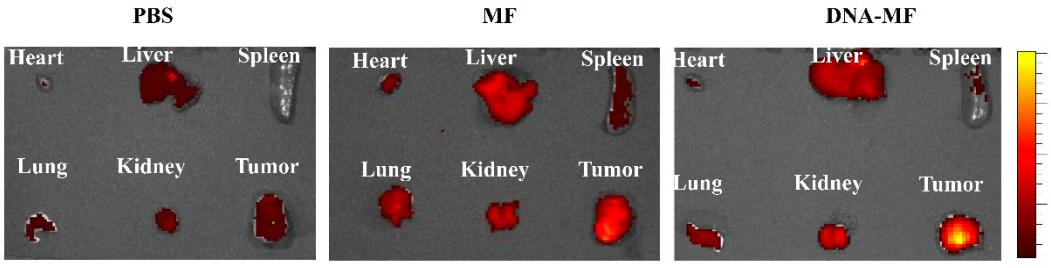

Fig. S32 Fluorescent images of tissues obtained from the mice, which were treated with PBS, MF and DNA-MF, respectively.

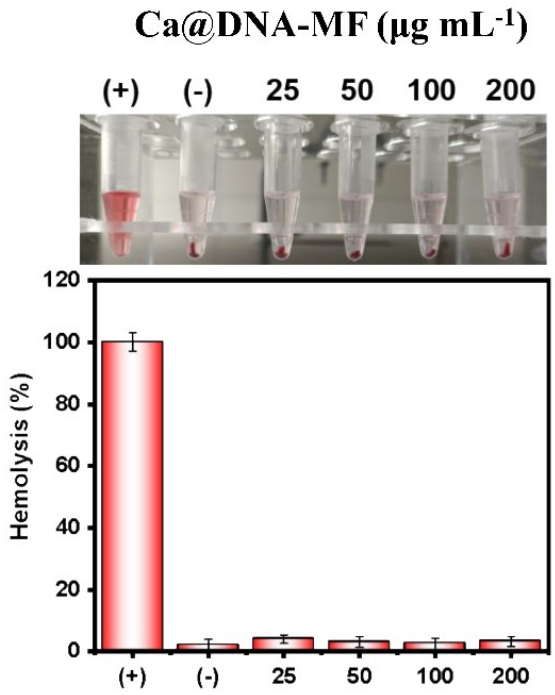

Fig. S33 Hemolysis test for Ca@DNA-MF at different concentrations.

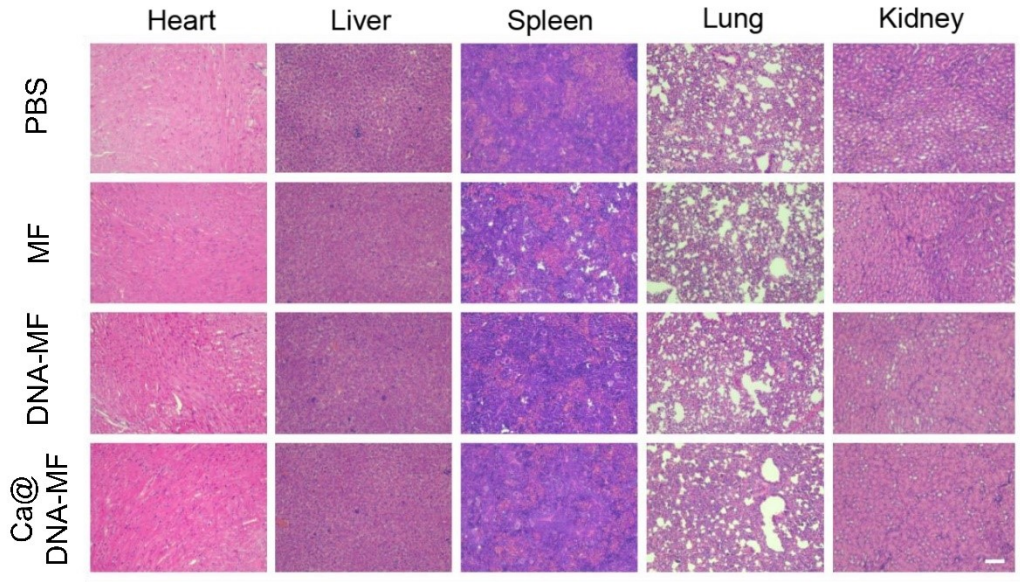

Fig. S34 Hematoxylin-eosin (H&E) staining of the major organs derived from the 4T1 tumor-bearing mice at the end of treatment. The heart, liver, spleen, lung, and kidney tissue slices were from tumor-bearing mice with different treatments for 14 days. Scale bars: 100 μm.

Table 1. DNA and siRNA Oligonucleotide sequences used in this work

Name	Sequences (5'-3')
N1-DNA	CTAGAGCACAATCACAGGAGCCAG
H1	GGATCGCGATCCCTGGCTCCTGTGATTGTGCTCTAGACA TCGCTAGAGCACAATCACAGGCTAGAGCACAATCACAGG AGCCAGTTTTCCTGTGATTGT
H2	GCTCTAGCGATGTACCTGGGGGAGTATTGCGGAGGAAG GTCTAGAGCACAATCACAGGAGCCAGTTTTCCTGTGATT GT
non-H2	GCTCTAGCGATGTATTTGACAAATTAAAGCGGAGGAAGG T CTAGAGCACAATCACAGGAGCCAGTTTTCCTGTGATTGT
TAMRA-H2	GCTCTAGCGATGTACCTGGGGGAGTATTGCGGAGGAAG GT-3`TAMRA- CTAGAGCACAATCACAGGAGCCAGTTTTCCTGTGATTGT
TAMRA-nATP-H2	GCTCTAGCGATGTATTTGACAAATTAAAGCGGAGGAAGG T-3`TAMRA
ssDNA	TGGCAGTGTCTTAGCTGGTTGTACCTTCCTCCGC
TAMRA-ssDNA	5`TAMRA- TGGCAGTGTCTTAGCTGGTTGTACCTTCCTCCGC
BHQ2-ssDNA	TGGCAGTGTCTTAGCTGGTTGTACC/iBHQ2dT/TCCTCCGC
siMCT4	5’- TCCCATGGCCAGGAGGGTTGACCTTCCACCGC-3’

Red sequences are “sticky ends” as siRNA linkers.

Reference

[1] K. Yan, H. Li, P. Li, H. Zhu, J. Shen, C. Yi, S. Wu, K. W. Yeung, Z. Xu, H. Xu, P. K. Chu, *Biomaterials* 2014, **35**, 344-355.

[2] H. Kong, Q. Chu, C. Fang, G. Cao, G. Han, X. Li, *Adv. Sci.* 2021, **8**, 2100241.

[3] P He, F. Liu, M. Li, M. Ren, X. Wang, Y. Deng, X. Wu, Y. Li, S. Yang, J.

Song, *Adv. Healthcare Mater.* 2023, **12**, 2203106.