



Stimuli-responsive magnetic black phosphorus-based nanoreactor for chirality-dependent anoikis induction in cancer therapy

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Abstract

Anoikis presents an alternative scheme for suppressing tumor metastasis. While limited by off-target effects, rapid degradation, and reactive oxygen species (ROS) accumulation, anoikis resistance remains a persistent challenge for current tumor therapy. The use of external and endogenous stimuli to trigger ROS bursts, as well as chirality-enhanced tumor microenvironment (TME) penetration, offers a valid solution. We developed a stimuli-responsive nanoreactor by coupling chiral histidine-modified manganese ferrite (MnFe₂O₄-PEG) nanoparticles with chiral cysteine-modified black phosphorus (BP) nanosheets via an ROS-sensitive thioketal (TK) bond to form a MnFe₂O₄-BP chiral magnetic nanoreactor (CT-MFBP). L-MFBP exhibited superior cellular uptake and intracellular retention due to its enhanced affinity for cell membranes. After entering the TME, the thioketal bond was cleaved by alternating magnetic field (AMF)-induced ROS, leading to the decomposition of the nanoreactor into chiral BP components. Subsequently, ROS accumulation was further amplified using BP-mediated photodynamic therapy (PDT). In addition, the nanoreactor demonstrated catalase (CAT)-like activity, converting endogenous H₂O₂ into O₂, thereby effectively alleviating the tumor hypoxic microenvironment and enhancing PDT efficacy. Furthermore, these events activated the mitogen-activated protein kinase (MAPK)-caspase 3 signaling pathway by upregulating p38 protein and Bcl-2-modifying factor (BMF) expression, which together promoted anoikis in tumor cells—a finding further validated by genomic analysis. Both in vitro and in vivo results confirmed that L-MFBP provides a robust regulatory strategy to induce anoikis and suppress tumor metastasis, offering a promising approach for chiral magnetic nanomedicine in clinical anti-tumor applications.

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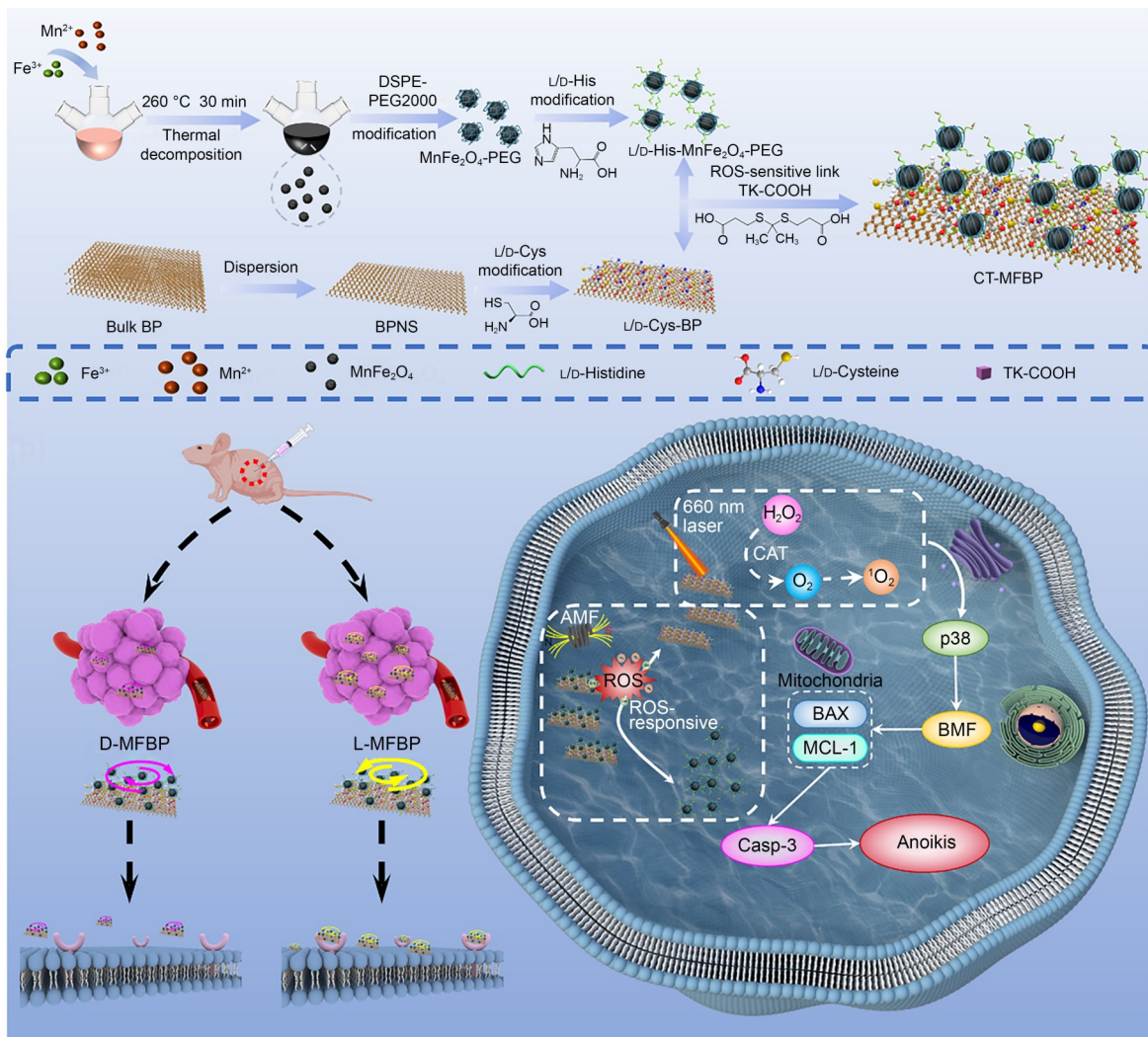
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Graphical abstract



Keywords Reactive oxygen species (ROS)-sensitive · Magnetic-laser stimuli-responsive · Chirality-dependent · Anoikis · Cancer therapy

1 Introduction

The complex pathological process of tumor progression—especially the resistance to anoikis upon detachment from the extracellular matrix (ECM)—severely hinders the efficacy of tumor treatment, particularly in terms of uncontrollable tumor metastasis and dissemination [1–5]. Under normal conditions, tumor cells can undergo anoikis, a specialized form of programmed cell death, when they detach from the ECM [6]. Anoikis is triggered by the loss of cell–ECM or cell–cell adhesion and serves as a crucial innate defense mechanism for maintaining organismal homeostasis [5–7]. Once tumor cells detach from the tumor microenvironment (TME), they

undergo anoikis, thereby preventing tumor metastasis. The initiation and execution of anoikis involve complex signaling pathways that are mediated primarily through the intrinsic apoptotic pathway. This pathway is characterized by the oligomerization of the pro-apoptotic protein Bcl-2-associated X protein (BAX) in the outer mitochondrial membrane, leading to the release of cytochrome c and the subsequent activation of the executioner caspase-3 (Casp-3), ultimately inducing cell death [8, 9]. The death receptor-mediated extrinsic pathway (such as the Fas/tumor necrosis factor receptor pathways) also participates in this process.

Upon detachment from the ECM, cells exhibit down-regulation of $\beta 1$ integrin and epidermal growth factor

receptor (EGFR), inhibition of extracellular signal-regulated kinase 1 (ERK1) signaling, and upregulation of the pro-apoptotic protein Bim, resulting in cell death [10, 11]. However, cancer cells evolve multiple mechanisms to acquire anoikis resistance during tumor progression, enabling them to survive after detachment from the primary tissue, thereby promoting rapid tumor growth and metastasis [12, 13]. While current cancer therapeutic technologies, such as chemotherapy [14] and radiotherapy, can inhibit tumor progression to some extent, most do not target the anoikis pathway. Consequently, their efficacy in promoting anoikis and inhibiting tumor metastasis remains unsatisfactory [15]. Therefore, targeting the anoikis pathway while simultaneously inhibiting anoikis resistance to forcibly eliminate tumor cells under adverse microenvironmental conditions provides a novel, potentially selective therapeutic strategy for suppressing tumor growth.

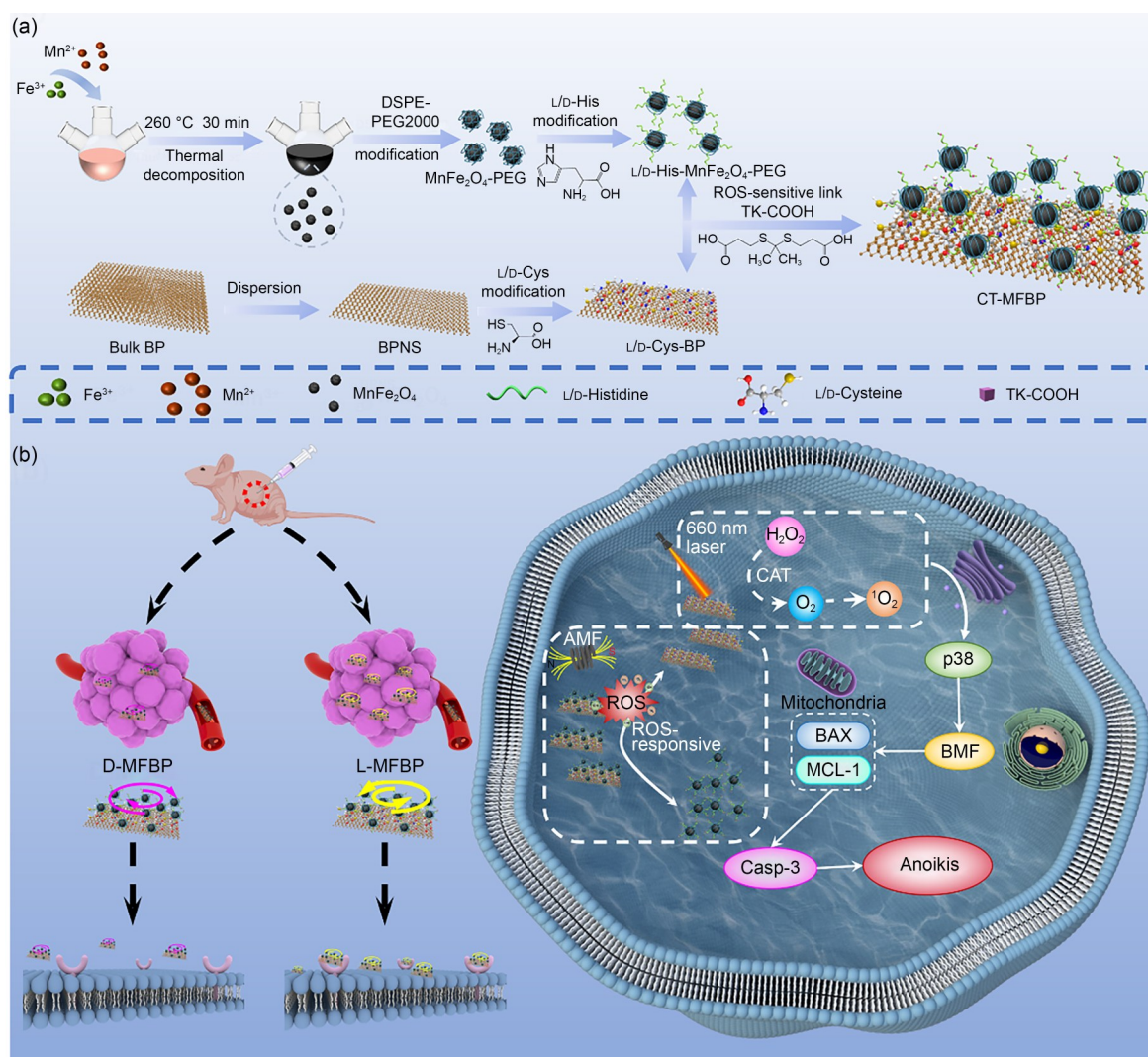
Emerging research on inducing anoikis to combat tumors has made preliminary progress across a wide range of approaches, from natural products to artificial small-molecule drugs [16]. For example, various plant extracts [17–19], fungal metabolites [20–23], and chemical derivatives [24–28] have been shown to induce anoikis in colorectal cancer cells. However, small-molecule drugs generally suffer from common bottlenecks, such as low bioavailability, off-target effects, rapid degradation, and difficulty penetrating dense tumor stroma [29]. Furthermore, multifunctional nanomaterials have been widely designed to target anoikis by generating reactive oxygen species (ROS) to inhibit key signaling pathways or enhance anoikis sensitivity, exhibiting precise tumor orientation and high biocompatibility. For instance, the MSN-Ce@SP/PEG nanoenzyme induces anoikis by generating ROS upon photothermal activation, resulting in mitochondrial damage, cellular apoptosis, and the inhibition of epithelial-mesenchymal transition (EMT) proteins in hepatocellular carcinoma cells [30]. Additionally, the nanoenzyme depletes intracellular glutathione (GSH), enhancing oxidative stress and cellular sensitivity to anoikis. However, these existing nanostrategies still face significant challenges [31]. On the one hand, the lack of on-demand, flexible regulation leads to inevitable toxicity and insufficient ROS accumulation. On the other hand, most designs neglect the interaction between the nanoagent and the TME; the transport and degradation properties of the nanoagent in the TME, which are crucial for subsequent anoikis regulation, remain unknown. Thus, a novel nanoplatform designed to address these challenges has proven to be a robust tool for breakthroughs in the induction of anoikis.

Nanomaterials with external physical-field or endogenous stimuli-responsive capabilities, such as magnetic [32] and laser [33] responsiveness, can generate abundant ROS in a controllable and facile manner. Distinguished from other catalytic ROS materials, these materials can enhance the precision of ROS formation in the tumor region while avoiding excess

ROS generation in healthy tissues, relying solely on magnetic and laser stimuli [34]. Furthermore, these nanomaterials exhibit excellent enzyme-like activities that participate in cellular biochemical reactions modulated by external stimuli, offering a potential therapeutic paradigm with high in vivo biosafety. In addition to the intrinsic enzyme-like activity, the surface properties of nanomaterials play a significant role in antitumor biological behavior. Chirality, a fundamental characteristic of biological systems [35], provides a unique regulatory dimension for the biointerface engineering of nanomaterials [36–38]. Studies have shown that chiral nanomaterials can interact with biological systems in a chirality-dependent manner determined by the special chiral type [39]; this phenomenon is expected to enhance the selectivity of nanomaterials for tumor cells while reducing potential toxicity to normal tissues, thereby improving biosafety [40, 41].

For example, Wang et al. [42] synthesized self-assembled copper-cysteine (Cys) nanoparticles with opposing chirality (Cu-L-Cys and Cu-D-Cys nanoparticles). The Cu-L-Cys nanoparticles demonstrated greater affinity for overexpressed L-GSH in cancer cells, leading to more efficient GSH depletion and enhanced hydroxyl radical generation via a Fenton-like reaction. This mechanism ultimately led to superior in vitro and in vivo tumor suppression compared with their D-counterpart. Additionally, Li et al. [43] developed L/D-arginine-modified multimodal nanoparticles (L/D-Cu/Z@MH). Cell transporters' natural preference for L-amino acids facilitates targeted delivery, amplifies chemokinetic therapy, and activates immune pathways. Consequently, the L-enantiomer-modified platform exhibits higher cellular uptake and enhanced antitumor efficacy. These studies suggested that chiral features play a key role in biological recognition and cellular responses for nanomaterials, which is especially promising for anoikis activation.

In the present study, we developed an intelligent stimuli-responsive nanoplatform by coupling chiral histidine (His)-modified manganese ferrite nanoparticles (MnFe_2O_4 -PEG) with chiral Cys-modified black phosphorus (BP) nanosheets linked through a thioketal (TK) bond sensitive to ROS in the TME, forming a MnFe_2O_4 -BP chiral magnetic nanoreactor (CT-MFBP) (Scheme 1a). First, due to its stronger affinity for cell membranes, L-MFBP achieves rapid cellular uptake and higher intracellular retention. Subsequently, by virtue of the magnetic enhancement effect, the TK bond is cleaved by ROS generated under an alternating magnetic field (AMF), causing the nanoplatform to decompose into chiral MnFe_2O_4 -PEG and chiral BP. Next, ROS accumulation is amplified via BP-mediated photodynamic therapy (PDT). In addition, CT-MFBP exhibits catalase (CAT)-like activity that converts H_2O_2 into O_2 , thereby relieving the hypoxic microenvironment while significantly enhancing the efficiency of PDT. More importantly, these events trigger the mitogen-activated protein kinase (MAPK)-Casp-3 signaling pathway [44]



Scheme 1 Schematic illustrations of (a) the preparation process of CT-MFBP and (b) the proposed mechanism of anoikis induction by CT-MFBP in colon cancer therapy

by upregulating p38 protein and Bcl-2-modifying factor (BMF) [45], which collectively promote anoikis in tumor cells, as also validated by genomic analysis (Scheme 1b). Our design aims to leverage the synergistic effect of external magnetic-laser stimuli and chirality-induced CT-MFBP to achieve targeted delivery and on-demand initiation, and, through sufficient ROS production, ultimately realize effective anoikis for tumor treatment. This provides a feasible regulatory strategy for circumventing anoikis resistance, holding great promise for antitumor nanomedicine in clinical applications.

2 Materials and methods

2.1 Materials

Iron (III) acetylacetonate ($\text{C}_{15}\text{H}_{21}\text{FeO}_6$, 98%) and manganese acetylacetonate ($\text{MnC}_{15}\text{H}_{21}\text{O}_6$, 97%) were purchased from

Aladdin (Shanghai, China) and used as precursors for material synthesis. 1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(poly(ethylene glycol)-2000) (DSPE-PEG2000) was obtained from Southeast Nano Technology Co., Ltd (Suzhou, China) and employed for surface modification of manganese ferrite nanoparticles. D/L-histidine (D/L-His, $\text{C}_6\text{H}_9\text{N}_3\text{O}_2$, 99%) and D/L-cysteine (D/L-Cys, $\text{C}_3\text{H}_7\text{NO}_2\text{S}$, 99%) were acquired from Aladdin. Black phosphorus crystals (BP, 99.998%) were supplied by Xianfeng Nano Co., Ltd. (Nanjing, China). 2,2'-(Propane-2,2-diylbis(thio))diacetic acid (TK-COOH, $\text{C}_7\text{H}_{12}\text{O}_4\text{S}_2$, 97%), used for reactor construction, was procured from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). 2,2,6,6-Tetramethylpiperidine-1-oxyl (TEMPO), 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), and cyanine 7 (Cy7) were purchased from Tokyo Chemical Industry (Shanghai) Development Co., Ltd. (Shanghai, China). 5,5'-Dithiobis (2-nitrobenzoic acid) (DTNB) and 1,3-diphenylisobenzofuran (DPBF) were bought from Macklin Biochemical Technology

Co., Ltd. (Shanghai, China). Phosphate-buffered saline (PBS; KGL2206-500), cell counting kit-8 (CCK-8; KGA9310-500), bicinchoninic acid (BCA) protein assay kit (KGB2101-250), and Roswell Park Memorial Institute (RPMI) 1640 medium (P/S, KGL1501-500) were acquired from KeyGEN BioTECH (Nanjing, China). Trypsin–ethylenediaminetetraacetic acid (EDTA) solution (BL512A) was purchased from Biosharp (Beijing, China). ROS assay kit-highly sensitive 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA; AP853) was obtained from Dojindo Laboratories (Kumamoto, Japan), and the mitochondrial membrane potential assay kit with 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1; M8650) was sourced from Solarbio (Beijing, China). The LIVE/DEAD cell imaging kit (R37601) was obtained from Invitrogen by Thermo Fisher Scientific (Beijing, China), and the Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (C1062L) was acquired from Beyotime (Shanghai, China).

2.2 Synthesis and surface modification of manganese ferrite (MnFe₂O₄-PEG)

Manganese acetylacetonate (Mn(acac)₃; 101.3 mg, 0.4 mmol) was combined with oleyl alcohol (4 mmol), oleic acid (2 mL), oleylamine (2 mL), and dibenzyl ether (24 mL) in a three-necked flask (50 mL). Concurrently, iron acetylacetonate (Fe(acac)₃; 282.5 mg, 0.8 mmol), was dissolved in dibenzyl ether (8 mL) in a centrifuge tube to form an Fe(acac)₃ solution. Nitrogen gas was continuously bubbled through the mixture for 30 min under mechanical stirring to eliminate oxygen and moisture. Subsequently, the flask was heated on a heating mantle to 140 °C at a ramp rate of 3 °C/min. The Fe(acac)₃ solution was slowly added with stirring, and the temperature was raised to 260 °C at 3 °C/min, resulting in a color change from reddish-brown to dark black. After maintaining the reaction under a nitrogen atmosphere for 30 min, the mixture was cooled to room temperature. The obtained MnFe₂O₄ dispersion was subjected to magnetic separation or washed thrice with anhydrous ethanol, followed by the addition of *n*-hexane (15 mL). The resulting dispersion was transferred to a glass bottle with a spiral mouth [46].

The MnFe₂O₄ dispersion (2 mg/mL Fe, 2 mL) was combined with a solution of DSPE-PEG2000 in CH₂Cl₂ (33 mg/mL, 3 mL) and subjected to ultrasonic treatment for 20 min. MnFe₂O₄-PEG was subsequently prepared through solvent evaporation. Upon dissolving in ultrapure water (2 mL), MnFe₂O₄ transitioned from the organic phase to the aqueous phase.

2.3 Synthesis of chiral MnFe₂O₄-PEG

The aqueous MnFe₂O₄-PEG solution (1 mL) was added to ultra-pure water (30 mL). D/L-His (8 mg) was then introduced,

and the mixture was stirred for 2 h at 50 °C under nitrogen. The solution was allowed to cool to room temperature, and then centrifuged and dried to obtain chiral histidine-modified MnFe₂O₄-PEG.

2.4 Preparation of BP nanosheets and chiral BP nanosheets

Small BP crystals (20 mg) were weighed and placed in a mortar. *N*-methyl-2-pyrrolidone (NMP, 1 mL) solution was added, and the mixture was ground for 20 min before being transferred to a centrifuge (50 mL) tube. Subsequently, the NMP (24 mL) solution was introduced, and nitrogen was allowed to flow for 15 min. The tube was then sealed with a sealing film and placed in an ice water bath. The cell was disrupted using an ultrasonic cell disruptor set to 200 W for 6 s, with a 2-s interval. The ice was changed every 30 min. After 24 h of ultrasonic treatment, the resulting brownish-yellow suspension was centrifuged at 5000 r/min for 20 min, and the supernatant was collected. It was centrifuged again at 11,500 r/min; the supernatant was discarded, and the resulting precipitate was retained as BP nanosheets. The precipitated BP nanosheets were added to ultrapure water prepared in advance, and centrifugation was continued at 11,500 r/min to wash away any remaining NMP. Finally, the precipitate was redispersed in ultrapure water (de-aerated in advance) to obtain the water dispersion solution of BP nanosheets. BP nanosheets were described in previous articles published by the laboratory [47].

D/L-Cys (15.6 mg) was dissolved in ultrapure water (10 mL). Subsequently, the prepared BP nanosheet dispersion (2 mg/mL, 1 mL) was added to the D/L-Cys aqueous solution and stirred for 20 min to obtain the D/L-Cys-BP water dispersion.

2.5 Synthesis of MnFe₂O₄-BP chiral magnetic nanoreactor (CT-MFBP)

TK-COOH (20 mg) was dissolved in *N,N*-dimethylformamide (DMF, 15 mL). Under continuous nitrogen flow, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, 60 µg) was added, and the solution was stirred for 15 min. Subsequently, *N*-hydroxysuccinimide (NHS, 24.8 mg) was added, and stirring was continued for another 45 min. The as-synthesized chiral MnFe₂O₄-PEG and chiral BP were then added to the mixture, which was mechanically stirred for 4 h. The product was rinsed with ultrapure water, centrifuged three times to remove impurities, and then dispersed in ultrapure water. For the control sample C-MFBP, the procedure remained identical except that no TK-COOH was added. Meanwhile, the non-chiral variant T-MFBP was prepared under otherwise identical conditions but using non-chirally modified MnFe₂O₄-PEG and BP. CT-MFBP was categorized into D-MFBP and L-MFBP. D-MFBP was formed when the

amino acids D-His and D-Cys were incorporated during the preparation process, whereas L-MFBP was produced when L-His and L-Cys were added.

2.6 Determination of CAT activity

C-MFBP (100 µg/mL Fe), T-MFBP (100 µg/mL Fe), and CT-MFBP (100 µg/mL Fe) were dispersed in 1× PBS by gentle stirring at 600 r/min. The change in oxygen solubility was monitored using a specific oxygen analyzer (Multi 3510IDS, Wissenschaftlich-Technische Werkstätten, Weilheim, Germany). Once the system stabilized, hydrogen peroxide (H₂O₂, 100 µL) solution was added at varying concentrations (10, 20, 50, 100, 200, 300, and 500 µmol/L). The concentration of oxygen produced was recorded every 10 s for 5 min. Subsequently, the data were fit to the Michaelis–Menten kinetic equation.

2.7 Detection of ¹O₂

DPBF exhibits a prominent absorption peak in the ultraviolet–visible (UV–Vis) spectrum at approximately 410 nm, prior to its reaction with singlet oxygen (¹O₂). However, upon interaction with ¹O₂, the absorption at 410 nm of the resultant product is markedly diminished. Consequently, DPBF serves as a probe for ¹O₂, allowing both qualitative and quantitative analysis of ¹O₂ generation by monitoring changes in the absorption peak at 410 nm. Initially, DPBF was dissolved in dimethyl sulfoxide (DMSO) to create a stock solution at a concentration of 1 mg/mL. The DMSO-diluted sample dispersion was then combined with the DPBF solution, treated to prevent light exposure, and subjected to ultrasonic mixing in the dark for 10 min. The samples were categorized into four groups: a control group that received no treatment, a group exposed to 660 nm laser irradiation, a group subjected to AMF, and a group that first received AMF, followed by 660 nm laser irradiation. Samples were collected every 3 min to assess the changes in UV–Vis spectral absorption from 300 to 600 nm, with measurements conducted over a 15-min period.

¹O₂ was detected using electron paramagnetic resonance (EPR). In aqueous solutions of C-MFBP (1 mg/mL Fe), T-MFBP (1 mg/mL Fe), and CT-MFBP (1 mg/mL Fe), the capture agent TEMPO (10 µL, 0.1 mmol/L) was rapidly added and mixed ultrasonically for 3 min. The samples were then divided into four groups: a control group with no treatment, a group subjected to 660 nm laser irradiation, a group exposed to AMF, and a group receiving AMF first, followed by 660 nm laser irradiation. Capillary samples were collected every 3 min and analyzed using EPR to monitor the appearance of peak shapes; this process lasted a total of 15 min.

2.8 Detection of ROS

ROS generation was assessed using DCFH-DA. A concentration of 0.5 mmol/L DCFH-DA was added to the aqueous solutions of C-MFBP (100 µg/mL Fe), T-MFBP (100 µg/mL Fe), and CT-MFBP (100 µg/mL Fe). Fluorescence intensity was measured every 3 min with a fluorescence spectrophotometer under four conditions: no effect, exposure to a 660 nm laser, application of AMF, and a group receiving AMF first, followed by 660 nm laser irradiation. This procedure lasted 21 min. ROS generation was determined by monitoring changes in peak intensity at 525 nm.

2.9 GSH depletion

The consumption of GSH was assessed utilizing DTNB as a probe. The disulfide bond in DTNB reacts with the free thiol group (–SH) of GSH to produce yellow 2-nitro-5-thiobenzoic acid (TNB) and oxidized glutathione (GSSG). TNB exhibits a distinct absorption peak at 412 nm, with absorbance showing a linear and positive correlation with GSH concentration. An enzyme-linked immunosorbent assay reader was employed to detect the absorbance change at 412 nm, enabling the calculation of the remaining GSH concentration. As GSH is consumed, less GSH remains, resulting in reduced TNB production and lower absorbance levels. The process involved adding 70 µL of material solutions (C-MFBP (500 µg/mL Fe), T-MFBP (500 µg/mL Fe), CT-MFBP (500 µg/mL Fe)), 30 µL of GSH (0.5 mmol/L) solution, and 4 µL of DTNB (0.2 mmol/L) to 96-well plates. Subsequently, a 660 nm laser, AMF, and a combination of both were applied for 15 min each. Samples were collected every 3 min, and the optical density (OD) value at 412 nm was measured using a microplate reader.

2.10 Cell culture

In vitro experiments were performed using SW480 human colon cancer cells. The cells were cultured in a controlled environment at 37 °C with a partial pressure of 5% CO₂. Following the initial inoculation in a culture dish containing 10% fetal bovine serum (FBS) and 90% RPMI 1640 for 24 h, the cells were treated with trypsin solution for 2–3 min. The digestion process was subsequently halted using high-glucose RPMI 1640 medium. After centrifugation, the cells were cultured at a specified concentration.

2.11 Cell biocompatibility assay

Immortalized human bladder epithelial cells (SV-HUC-1) and human endothelial cells (ECs) were cultured in 96-well plates with 100 µL per well (1×10⁵ cells) for 24 h. Following cell adhesion, media containing varying concentrations

of materials (0, 6.25, 12.5, 25, 50, and 100 $\mu\text{g/mL}$) were added and incubated for an additional 24 h. Subsequently, the culture media were aspirated, wells were washed twice with PBS, and then each well was supplemented with CCK-8 (10 μL) and culture medium (990 μL) for a 1-h incubation. Finally, the absorbance of the solution in each well was determined at 450 nm with a microplate reader.

2.12 Cell viability assay

Cell survival rates were assessed using the CCK-8 method. Cells were cultured in an incubator at a density of 100 μL per well (1×10^5 cells) for 24 h, allowing them to adhere to the 96-well plate. Once the cells were attached, media containing varying concentrations of materials (0, 6.25, 12.5, 25, 50, and 100 $\mu\text{g/mL}$) were added and incubated for an additional 24 h. Subsequently, the media were aspirated, and the wells were washed twice with PBS. Following this, CCK-8 (10 μL) reagent and medium (990 μL) were added to each well and incubated for 1 h. Finally, the absorbance values of the solutions in each well were measured at 450 nm.

2.13 Cell live/dead assay

SW480 cells were cultured for 24 h in a 35 mm culture dish at a density of 1×10^6 cells. The cells were divided into seven groups: Con, T-MFBP, D-MFBP, L-MFBP, T-MFBP+AMF+Las, D-MFBP+AMF+Las, and L-MFBP+AMF+Las. Following the addition of materials and exposure to various stimuli, the cells were cultured for an additional 6 h. After removing the culture medium, the cells were washed twice with PBS. Subsequently, calcein acetoxymethyl ester (Calcein-AM) and BOBO-3 solutions (1 mL) prepared in culture medium were added, and the cells were incubated in the dark at 37 °C for 45 min. The cells were then washed three times with PBS. Fluorescence images were captured using confocal laser scanning microscopy (CLSM), and the relative fluorescence intensity of each image was quantified using ImageJ.

2.14 Mitochondrial membrane potential assay

SW480 cells were cultured in a 35 mm culture dish at a density of 1×10^6 for 24 h. The cells were allocated into seven groups: Con, T-MFBP, D-MFBP, L-MFBP, T-MFBP+AMF+Las, D-MFBP+AMF+Las, and L-MFBP+AMF+Las. Following the addition of materials and exposure to various stimuli, the cells were cultured for an additional 6 h. After removing the culture medium, the cells were washed twice with PBS. Subsequently, JC-1 from the mitochondrial membrane potential kit (1 mL) was added, and the cells were treated in the dark at 37 °C for 20 min. The cells were then washed three times with PBS. Fluorescence images were captured using CLSM, and the relative fluorescence intensity of each image was quantified using ImageJ.

2.15 ROS level measurement

Cells were cultured in 35 mm dishes at a density of 1×10^6 cells per dish for 24 h. The cells were then divided into seven groups: control (Con), T-MFBP, D-MFBP, L-MFBP, T-MFBP+AMF+Las, D-MFBP+AMF+Las, and L-MFBP+AMF+Las. After adding materials and various stimuli, the cells were cultured for an additional 6 h. After removing the culture medium, the cells were washed three times with PBS and treated with DCFH-DA solution diluted in PBS (1 $\mu\text{mol/L}$, 1 mL). The cells were incubated in the dark at 37 °C for 60 min, and then washed three times with PBS. Finally, confocal fluorescence imaging was performed to capture images, and the relative fluorescence intensity of each image was quantified using ImageJ.

2.16 Apoptosis detection by flow cytometry

Cells were cultured in 35 mm dishes at a density of 1×10^6 per dish for 24 h. The cells were divided into seven groups: Con, T-MFBP, D-MFBP, L-MFBP, T-MFBP+AMF+Las, D-MFBP+AMF+Las, and L-MFBP+AMF+Las. After mixing the cells with the respective materials and applying various stimuli, they were cultured continuously for an additional 12 h. The cell culture medium from each group was collected, and the cells were digested using pancreatic enzymes devoid of EDTA. The collected cell culture medium and cell suspension were then transferred to an EP tube, centrifuged at 1000 r/min for 5 min, and the supernatant was discarded. The cells were washed twice with PBS, centrifuged again, and the PBS was discarded. Subsequently, binding buffer (195 μL) was added to each tube. Prior to analysis, Annexin V-FITC (5 μL) was added to each tube for a 5-min staining period, followed by the addition of propidium iodide (PI) staining solution (10 μL) for another 5 min. Detection was performed using a flow cytometer, and the data were analyzed with FlowJo.

2.17 Cellular uptake

Cells were cultured in 35 mm dishes at a density of 1×10^6 cells per dish for 24 h. The cells were allocated into seven groups: Con, T-MFBP, D-MFBP, L-MFBP, T-MFBP+AMF+Las, D-MFBP+AMF+Las, and L-MFBP+AMF+Las. Following the addition of materials and exposure to various stimuli, the cells were cultured for an additional 24 h. After the culture medium was removed, the cells were washed three times with PBS and subsequently imaged in bright-field mode using CLSM.

2.18 Inductively coupled plasma-mass spectrometry (ICP-MS) detection

The concentration of D/L-MFBP internalized by SW480 cells was quantified using ICP-MS analysis. SW480 cells were

cultured for 24 h in 35 mm culture dishes at a density of 1×10^6 cells per dish. Subsequently, the cells were incubated for 12 h with D/L-MFBP at 100 $\mu\text{g/mL}$, replacing the cell culture medium, and then washed three times with PBS. To eliminate D/L-MFBP adsorbed to the cell surface, the cells were removed and rinsed a further three times with PBS. Following this, 200 μL of concentrated nitric acid was added, and the mixture was heated in a 60 $^{\circ}\text{C}$ water bath for 6 h. Deionized water was then added to dilute the total volume to 2 mL. The concentrations of iron and phosphorus in the sample were subsequently measured using ICP-MS.

2.19 Isothermal titration calorimetry (ITC) measurement

The ITC experiments were performed using a NANO ITC instrument (TA Instruments, Delaware, USA) at 25 $^{\circ}\text{C}$. Prior to measurement, all samples were degassed under vacuum for 10 min. The sample cell was loaded with 300 μL of cell membrane solution (0.3 mmol/L), and the syringe was filled with 60 μL of ligand solution (Material 1 or Material 2, 2 nmol/L). The titration protocol involved 20 successive injections with a 200 s spacing and a constant stirring speed of 500 r/min. Heat changes upon each injection were recorded in real time. Binding isotherms were generated by integrating the heat peaks and normalizing for concentration. Data were fitted using the NanoAnalyzer software (TA Instruments) with an appropriate binding model to derive the dissociation constant (K_d), stoichiometry (N_0), enthalpy change (ΔH), and entropy change (ΔS).

2.20 Construction of subcutaneous tumor-bearing mouse models

SW480 cells were injected subcutaneously into the left axilla of 4-week-old male BALB/c nude mice to establish a subcutaneous tumor-bearing model. Tumor length (L_T) and width (W_T) were measured every 2 d using a digital vernier caliper. Tumor volume was calculated using the formula: $(L_T \times W_T^2)/2$. The subcutaneous tumor model of colon cancer was deemed successfully established when the tumor volume reached 90–100 mm^3 , which allowed for subsequent experiments.

2.21 In vivo antitumor experiments

All animal experiments were performed following the guidelines for the care and use of laboratory animals established by the Affiliated Hospital of Integrated Traditional Chinese and Western Medicine, Nanjing University of Chinese Medicine. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Hospital (Ethical Lot Number: 2022-LWKYS-046). Mice were randomly assigned to seven treatment groups: Con, T-MFBP, D-MFBP, L-MFBP,

T-MFBP+AMF+Las, D-MFBP+AMF+Las, and L-MFBP+AMF+Las. A total of 100 μL (100 $\mu\text{g/mL}$ Fe) was administered four times at 3-d intervals. Following intratumoral injection, each group received a magnetic field and an appropriate laser treatment for 20 min. The laser intensity at 660 nm was set at 0.22 W/cm^2 , while the magnetic field intensity was maintained at 210 μT . Throughout the treatment period, tumor volume and mouse weight were recorded every 2 d, and photographs were taken. All mice were euthanized following 14 d of treatment. Major organs, including the kidney, liver, spleen, heart, and lung, along with tumors, were collected and preserved in 4% fixative. Histological evaluations were conducted using hematoxylin and eosin (H&E) staining, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, and Ki-67 immunofluorescence analysis.

2.22 Western blotting

Following the sacrifice and dissection of the mice, tumor tissues were promptly placed in liquid nitrogen and subsequently stored at -80°C . The entire Western blot sampling procedure was conducted on ice. Tumor tissues from the seven groups of mice were first minced into small pieces using scissors. Protease inhibitor (20 μL), phosphatase inhibitor (20 μL), EDTA (20 μL), and radioimmunoprecipitation assay (RIPA) lysis buffer (200 μL) were then added, and the mixture was lysed at 4 $^{\circ}\text{C}$ for 30 min. After lysis, the samples were centrifuged at 4 $^{\circ}\text{C}$ and 12,000 r/min for 20 min. The precipitate was discarded, and the supernatant was collected. The protein concentration was determined using the BCA protein concentration assay kit. Subsequently, the protein concentration of each group was adjusted to 2 $\mu\text{g}/\mu\text{L}$ using RIPA lysis buffer (medium) and 5 $\times$ protein loading buffer, and the samples were heated in a 100 $^{\circ}\text{C}$ metal bath for 5 min. Finally, 10 μL of protein was loaded into each well for sodium dodecyl sulfate polyacrylamide gel electrophoresis, followed by membrane transfer, antibody incubation, and other procedures to obtain the target protein bands for BMF, p38, and p-p38.

2.23 In vivo imaging

For in vivo tracking, the material was labeled with the near-infrared dye Cy7 via chemical conjugation. The conjugate was then intratumorally injected into mice, and fluorescence imaging was performed at scheduled time points (0, 10, 20, 40, 60, and 120 min post-injection) to monitor its distribution and retention at the tumor site.

2.24 Transcriptome RNA-Seq analysis

Tumor tissues were collected from the in vivo colorectal cancer model after the designated treatment period. The treatment groups were divided into Con, T-MFBP, D-MFBP,

L-MFBP, T-MFBP+AMF+Las, D-MFBP+AMF+Las, and L-MFBP+AMF+Las. Tumor tissues were placed in cryogenic bottles, labeled according to their respective groups, rapidly frozen in liquid nitrogen for 2 min, and subsequently stored at -80°C until sequencing was required. Samples were submitted to GENEWIZ, Inc. (Suzhou, China) for transcriptome sequencing and analysis.

2.25 Data processing and dimensionality reduction

The raw RNA sequencing count data were processed and analyzed in R (version 4.2.0). To reduce dimensionality and facilitate visualization, uniform manifold approximation and projection (UMAP) was conducted utilizing the ‘umap’ package. Before UMAP analysis, count data were normalized using a variance-stabilizing transformation (VST) in DESeq2 to address variance–mean relationships. The UMAP visualization compared expression patterns among seven experimental groups: Con, T-MFBP, D-MFBP, L-MFBP, T-MFBP+AMF+Las, D-MFBP+AMF+Las, and L-MFBP+AMF+Las, each with three biological replicates.

2.26 Differential expression analysis

Differential gene expression analysis was conducted using the DESeq2 package (version 1.34.0) and involved three comparisons: Con vs. L-MFBP, L-MFBP vs. L-MFBP+AMF+Las, and D-MFBP+AMF+Las vs. L-MFBP+AMF+Las. Genes exhibiting an adjusted p -value <0.05 and an absolute $\log_2$ fold change >1 were deemed significantly differentially expressed. Volcano plots were generated with ggplot2 to visualize the distribution of differentially expressed genes (DEGs), with orange points indicating significantly upregulated genes, blue points representing downregulated genes, and gray points denoting non-significant changes. Expression patterns of key apoptosis-related genes were illustrated using a heatmap created with the pheatmap package. The expression values were scaled row-wise, and hierarchical clustering was performed on both rows and columns utilizing Euclidean distance and complete linkage. The corresponding single-cell data in .h5 format and annotation results were downloaded from TISCH. The R packages MAESTRO and Seurat were employed to process and analyze the single-cell data, followed by re-clustering using the t-SNE method.

2.27 Functional enrichment analysis

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were conducted utilizing the clusterProfiler package. The analysis employed significant DEGs from the D-MFBP+AMF+Las vs. L-MFBP+AMF+Las comparison as input. Gene symbols were converted to

ENTREZ IDs using the org.Hs.eg.db annotation package. GO enrichment analysis focused on biological process (BiP) terms, whereas KEGG analysis used the human pathway database.

2.28 Protein–protein interaction (PPI) network analysis

To explore the potential functional interactions among DEGs, we constructed a PPI network utilizing the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database (version 12.0, <https://string-db.org/>). The identified DEGs were entered into the STRING database to obtain their corresponding PPIs. We established the minimum required interaction score at medium confidence (0.400) and excluded disconnected nodes from the network. The resulting PPI network was visualized, with nodes representing proteins and edges denoting functional associations.

2.29 Statistical analysis

All quantitative data are presented as mean $\pm$ standard deviation (SD). Statistical analyses were performed using GraphPad Prism software (version 10.1.2, GraphPad Software, San Diego, CA, USA). One-way analysis of variance (ANOVA) was applied to compare three or more groups, followed by Tukey’s multiple comparisons test to identify specific group differences. When the experimental design involved two independent variables, data were analyzed using a two-way ANOVA, followed by Tukey’s multiple comparisons test for post-hoc analysis. A p -value of less than 0.05 was considered statistically significant. Significance levels in the figures are denoted as follows: * $p<0.05$, ** $p<0.01$, *** $p<0.001$, and **** $p<0.0001$.

3 Results and discussion

3.1 Synthesis and characterization of CT-MFBP

Manganese ferrite–black phosphorus chiral magnetic nanoreactor (CT-MFBP) was synthesized via multi-step chemical coupling. First, manganese ferrite (MnFe_2O_4) nanoparticles were synthesized via high-temperature decomposition in the oil phase, as previously reported by our lab [46] (Fig. S1a in the supplementary information), and their surface ligands were then exchanged with DSPE-PEG2000 to facilitate transfer into the aqueous phase and enhance biocompatibility and availability in vivo. BP nanosheets were prepared by liquid exfoliation, also previously reported by our lab [46] (Fig. S1b in the supplementary information). Subsequently, L/D-His was further modified onto MnFe_2O_4 -PEG, and L/D-Cys was conjugated to BP, after which ROS-sensitive TK bonds were used to link the MnFe_2O_4 -PEG and BP components, resulting in the formation of CT-MFBP. For comparison, C-MFBP

(Fig. S1c in the supplementary information) and T-MFBP (Fig. S1d in the supplementary information) were prepared using identical procedures but without chiral amino acids and TK-COOH components, respectively.

CT-MFBP exhibits a composite micromorphology of MnFe_2O_4 -PEG nanoparticles cluster loaded on BP nanosheets, with a size of approximately 700–800 nm (Fig. 1a). MnFe_2O_4 -PEG exhibits a cluster sphere with a regular shape and a size of approximately 100 nm (Fig. 1a; Fig. S1a in the supplementary information). The high-resolution transmission electron microscopy (HRTEM) images (Fig. 1b) reveal that the characteristic lattice fringes correspond closely to the (020) lattice planes in BP crystals and the (400) and (222) lattice planes in MnFe_2O_4 , suggesting tight coupling between BP and MnFe_2O_4 -PEG. Elemental mapping demonstrated that Fe and Mn elements were uniformly distributed throughout. P and S elements exhibit enrichment in a localized region, coinciding with the Fe/Mn signal region, indicating the successful construction of CT-MFBP (Fig. 1c). Atomic force microscopy (AFM) images demonstrate that the BP nanosheets are approximately 4 nm thick, while the height of the MnFe_2O_4 -PEG particles is around 6 nm (Fig. S2 in the supplementary information). Following compounding, CT-MFBP exhibits a “lamellar-particle” hierarchical structure; the particles adhere stably to the lamellar surface (Figs. 1d and 1e), forming a composite interface with highly active sites. The zeta potential values of MnFe_2O_4 -PEG, C-MFBP, T-MFBP, and CT-MFBP were -23.6 , 23.2 , -0.22 , and 25.5 mV, respectively. In contrast, the positive surface charges of C-MFBP and CT-MFBP are primarily due to the ionization of cysteine's amino groups, which facilitate the internalization of nanoparticles into cancer cells (Fig. 1f).

Dynamic light scattering (DLS) analysis revealed that the hydrodynamic size of MnFe_2O_4 -PEG was approximately 100 nm, with a polydispersity index (PDI) of 0.328, while the particle size of the assembled CT-MFBP rose to about 720 nm (PDI=0.361) (Fig. 1g), confirming their uniform dispersion and consistent with the scanning electron microscopy (SEM)/transmission electron microscopy (TEM) results. To further demonstrate the interaction between MnFe_2O_4 -PEG and BP, Fourier transform infrared (FTIR) spectra of C-MFBP, T-MFBP, and CT-MFBP were compared. The results showed that CT-MFBP exhibited characteristic absorption peaks at 2510 cm^{-1} (Cys S–H), 1730 cm^{-1} (PEG C=O), 1635 cm^{-1} (amide bond C=O), 1100 cm^{-1} (C–O–C), 805 cm^{-1} (TK-COOH C–S), and 578 cm^{-1} (Fe–O), along with a peak at 420 cm^{-1} (Mn–O). These findings confirm the complete chemical structure and successful surface modification of CT-MFBP (Fig. 1h). In the Raman spectrum, CT-MFBP exhibits P–O vibration peaks at 363, 440, 467 cm^{-1} , as well as Fe–O bonding signals at 607 cm^{-1} (Fig. 1i). The redshifted characteristic peak indicates the presence of lattice strain, which may be attributed to interface interactions. The weak peaks

at 445 and 540 cm^{-1} are attributed to the in-plane and out-of-plane swing vibrations of the O–C=O group in Cys, respectively. The peak at 670 cm^{-1} corresponds to the C–S vibration in Cys and thioketal. Furthermore, the sharp peak at 500 cm^{-1} corresponds to the S–S stretching vibration, indicating the inevitability of the oxidative transformation of Cys in air.

The X-ray diffraction (XRD) patterns further demonstrate the composite nanostructure. The prepared C-MFBP, T-MFBP, and CT-MFBP exhibited peaks at 18.2° (111), 30.0° (220), 35.3° (311), 37.0° (222), 42.9° (400), 53.3° (422), 56.8° (511), and 62.3° (440), which matched well with the crystal form of MnFe_2O_4 (PDF#01-071-4919) [48]. The peaks at 16.9° (002) and 34.2° (004) matched well with the crystal form of P (PDF#00-002-1020) (Fig. 1j). UV–Vis spectra showed that MnFe_2O_4 -PEG exhibited a broad absorption band, whereas the other three materials displayed no significant ultraviolet absorption. Upon surface modification, the absorption intensity of these materials gradually decreased, indicating altered optical properties (Fig. S3 in the supplementary information). Hysteresis loop tests using a physical property measurement system (PPMS) demonstrate typical superparamagnetism; the saturation magnetization of CT-MFBP (0.06 emu/g) is similar to that of MnFe_2O_4 -PEG and surpasses that of C-MFBP (0.003 emu/g) and T-MFBP (0.004 emu/g), suggesting that CT-MFBP has the most ordered magnetic structure, which remained intact throughout the reaction process (Fig. 1k).

Circular dichroism (CD) spectroscopy confirmed the successful preparation of chiral MnFe_2O_4 -PEG and chiral BP (Fig. S4 in the supplementary information). Figure 1l illustrates the successful construction of L-type and D-type CT-MFBP. L-MFBP demonstrated a positive Cotton effect at 220 nm, while the D-MFBP displayed symmetrical negative peaks at the same wavelength. X-ray photoelectron spectroscopy (XPS) analysis identified the presence of Fe-, Mn-, and P-containing components in C-MFBP, T-MFBP, and CT-MFBP, as illustrated in Fig. S5 (supplementary information). Specifically, the P 2p spectrum (Fig. 1m) can be deconvoluted into double peaks at approximately $130.1\text{ eV}/131.0\text{ eV}$, corresponding to P^0 , and additional double peaks at around $133.2\text{ eV}/134.1\text{ eV}$, associated with the P–O bond [49]. The Fe 2p spectrum (Fig. 1n) confirms the existence of a mixed valence $\text{Fe}^{2+}/\text{Fe}^{3+}$ state within these materials. Additionally, the $\text{Fe}^{2+}/\text{Fe}^{3+}$ ratio remained relatively constant. The Mn 2p spectrum in Fig. 1o reveals two peaks at 652.7 and 640.8 eV , with a spin-energy separation of 11.9 eV , aligning well with the binding energies of Mn $2p_{1/2}$ and Mn $2p_{3/2}$, respectively, indicating that Mn primarily exists in the Mn^{2+} oxidation state. Furthermore, the binding energies of Fe and Mn in C-MFBP, T-MFBP, and CT-MFBP increase progressively, suggesting that CT-MFBP possesses a more uniform composite structure with enhanced electronic interaction compared with the other two materials.

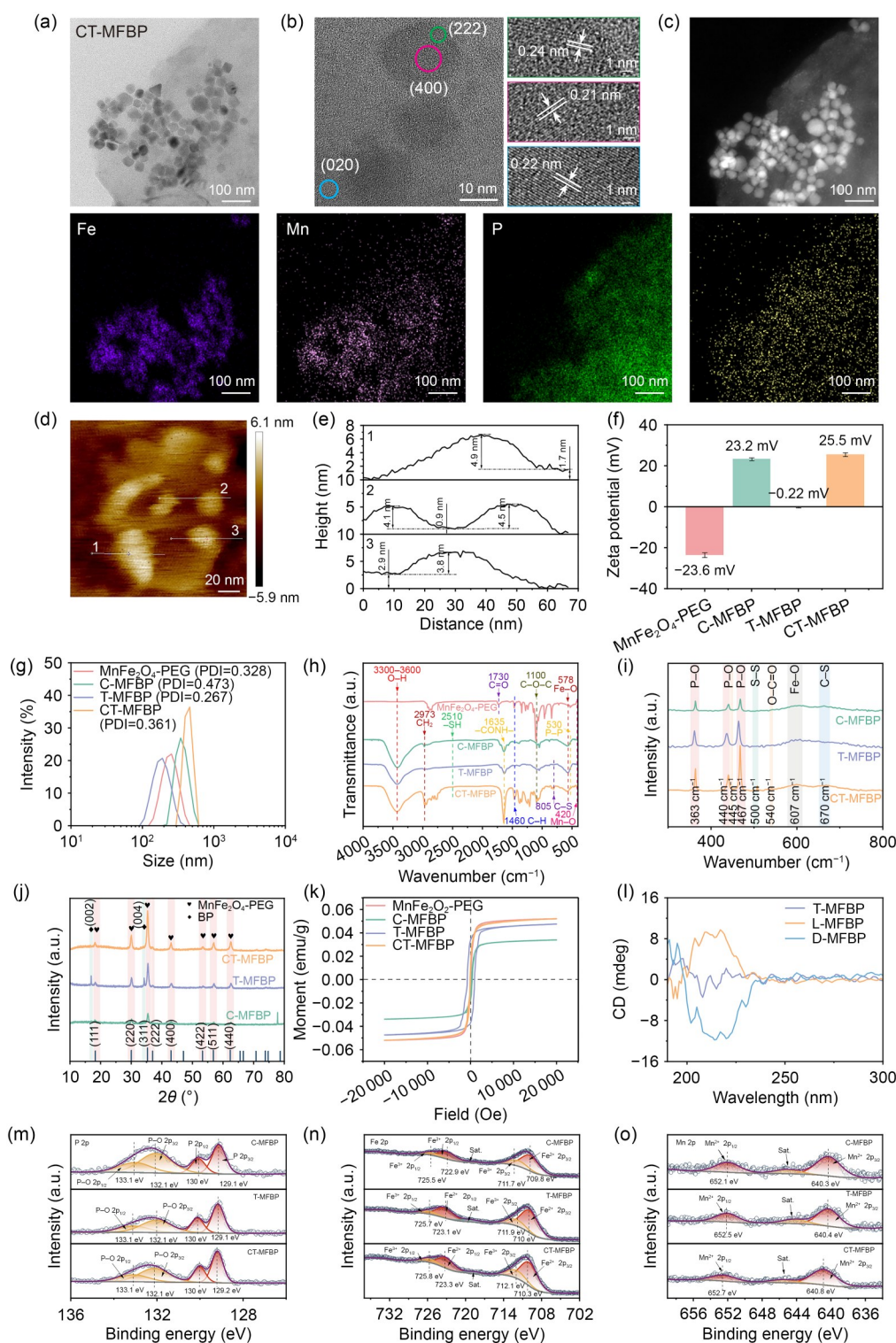


Fig. 1 Synthesis and structural characterizations of the nanoreactor. (a) Transmission electron microscopy (TEM) image of CT-MFBP. (b) High-resolution transmission electron microscopy (HRTEM) images of CT-MFBP. (c) Elemental mapping of CT-MFBP. (d) Atomic force microscopy (AFM) morphology image of CT-MFBP. (e) Height profile measured along the white lines in (d). (f) Zeta potential of MnFe₂O₄-PEG, C-MFBP, T-MFBP, and CT-MFBP. (g) Hydrodynamic size distribution of MnFe₂O₄-PEG, C-MFBP, T-MFBP, and CT-MFBP. (h) Fourier transform infrared (FTIR) spectra of MnFe₂O₄-PEG, C-MFBP, T-MFBP, and CT-MFBP. (i) Raman spectra of C-MFBP, T-MFBP, and CT-MFBP. (j) X-ray diffraction (XRD) patterns of C-MFBP, T-MFBP, and CT-MFBP. (k) Magnetic hysteresis loops of MnFe₂O₄-PEG, C-MFBP, T-MFBP, and CT-MFBP measured at room temperature. 1 emu/g=1000 A/m; 1 Oe=1000/(4 π) A/m=79.5775 A/m. (l) Circular dichroism (CD) spectra of L-MFBP, D-MFBP, and T-MFBP. (m–o) High-resolution X-ray photoelectron spectroscopy (XPS) results of C-MFBP, T-MFBP, and CT-MFBP: P 2p (m), Fe 2p (n), and Mn 2p (o) orbitals. Data are expressed as mean $\pm$ standard deviation ($n=3$)

3.2 Magnetic-laser stimuli response performance of CT-MFBP

The intrinsic enzyme-like activities and ROS generation performance under magnetic-laser stimuli were then analyzed. As shown in Fig. S6a (supplementary information), at identical H_2O_2 concentrations, CT-MFBP exhibited the highest oxygen generation, 1.5 times greater than that of C-MFBP and T-MFBP, indicating the strongest CAT activity. Figures S6b–S6d (supplementary information) demonstrate the concentration dependence of C-MFBP, T-MFBP, and CT-MFBP in response to H_2O_2 . Michaelis–Menten kinetics analysis (Fig. 2a) further indicated that CT-MFBP possesses the highest maximum reaction rate ($V_{\max}=29.6 \mu\text{mol}\cdot\text{L}^{-1}\cdot\text{s}^{-1}$) and the lowest Michaelis constant ($K_m=26.54 \text{ mmol/L}$), un-

derscoring its superior intrinsic catalytic efficiency and strong affinity for H_2O_2 . This robust oxygen generation capability provides a solid foundation for subsequent PDT applications. The response of these nanomaterials to stimuli was systematically evaluated, focusing on their catalytic performance and ROS generation under sequential magnetic and then laser stimulation (Fig. S7 in the supplementary information). The amount of singlet oxygen ($^1\text{O}_2$) generated by CT-MFBP under AMF is 2.9 times that without magnetic field treatment, facilitating the breakage of ROS-sensitive TK and then promoting subsequent ROS generation. As shown in Fig. 2b, the generation of $^1\text{O}_2$ detected by the DPBF fluorescence probe exhibited a time-dependent pattern. This trend is consistent with the quantitative results displayed in Fig. 2c, where CT-MFBP, subjected to sequential AMF stimulation followed by

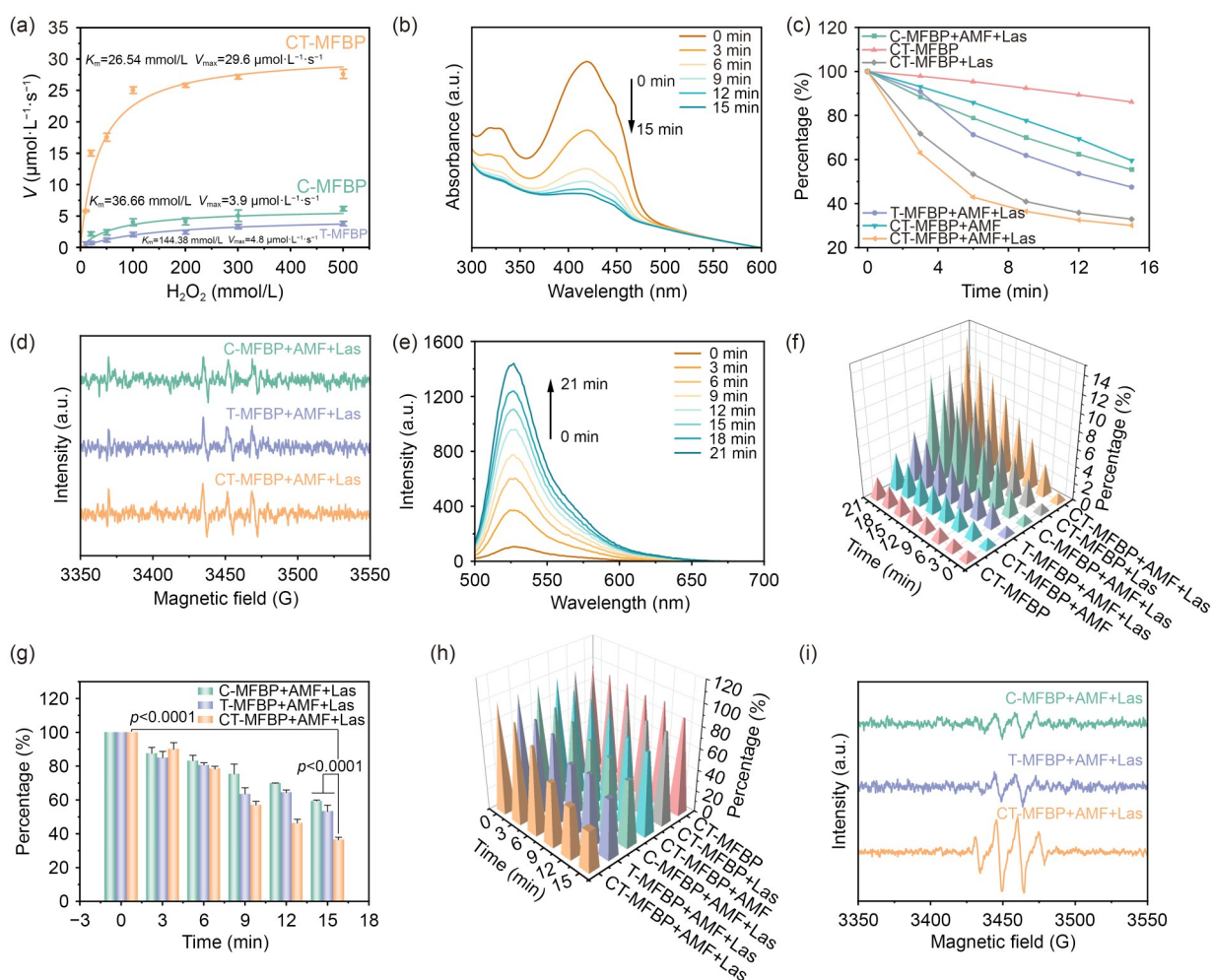


Fig. 2 Catalytic properties and reactive oxygen species (ROS) modulation performance of the nanoreactors. (a) Michaelis–Menten curves depicting the catalase (CAT)-like activities of C-MFBP, T-MFBP, and CT-MFBP. (b) Quantification of 1,3-diphenylisobenzofuran (DPBF) consumption by CT-MFBP under a sequential AMF+Las stimulus. (c) Normalized DPBF consumption curves for different materials under various stimuli (Las: laser only; AMF: magnetic field only; AMF+Las: magnetic field followed by laser). (d) Electron paramagnetic resonance (EPR) spectra for detecting $^1\text{O}_2$ generation by different materials under AMF+Las stimulus. (e) Quantification of total ROS generation by CT-MFBP under AMF+Las stimulus. (f) Normalized total ROS generation by different materials under various stimuli. (g) Glutathione (GSH) depletion by different materials under the AMF+Las stimulus. (h) Normalized GSH consumption by different materials under various stimuli. (i) EPR spectra for detecting $\cdot\text{OH}$ generation by different materials under the AMF+Las stimulus. Data are expressed as mean $\pm$ standard deviation ($n=3$)

660 nm laser irradiation (AMF+Las), achieved the highest $^1\text{O}_2$ production rate of 70%. This value was 1.56-fold and 1.33-fold greater than those of C-MFBP and T-MFBP, respectively, under the same conditions. $^1\text{O}_2$ generation by these materials under different conditions was detected by EPR (Fig. 2d; Fig. S8 in the supplementary information).

Corroborating the DPBF results, EPR spectroscopy provided direct evidence of $^1\text{O}_2$ generation, characterized by the distinctive 1:1:1 triplet signal at 3450 G. The most intense signal was observed for the CT-MFBP group subjected to sequential magnetic-laser stimulation, affirming its superior $^1\text{O}_2$ production capability. To further elucidate the catalytic mechanism, the ROS generation capacity of nanomaterials under various stimulation conditions was assessed using the DCFH-DA fluorescent probe (Fig. S9 in the supplementary information). The results show that under AMF, the amount of ROS generated by CT-MFBP is 1.7 times that without magnetic field treatment. Consistent with the $^1\text{O}_2$ generation results, TK bond cleavage occurs upon ROS attack, leading to the decomposition of the nanoreactor into MnFe_2O_4 -PEG and BP. Furthermore, a time-dependent increase in ROS generation was observed under the sequential stimulation protocol (Fig. 2e). CT-MFBP subjected to AMF+Las stimulation for 21 min produced 13.8-fold more ROS than the initial value, significantly outperforming C-MFBP and T-MFBP under identical conditions (Fig. 2f).

Furthermore, the capacity of the resultant antioxidant GSH to deplete was investigated [50]. When subjected to the AMF+Las, CT-MFBP demonstrated superior activity, depleting approximately 63.5% of GSH (Figs. 2g and 2h). This efficiency surpassed that of C-MFBP and T-MFBP by 1.56 and 1.36 times, respectively. In addition, EPR spectroscopy revealed hydroxyl radicals ($\cdot\text{OH}$) generation, shown in Fig. 2i and Fig. S10 (supplementary information), as identified by the characteristic 1:2:2:1 quartet signal of the DMPO- $\cdot\text{OH}$ adduct [51]. This signal was most pronounced for CT-MFBP under sequential AMF+Las stimulation, suggesting the presence of an alternative $\cdot\text{OH}$ generation pathway. Furthermore, the ROS-responsive microstructure was detected by SEM. As shown in the SEM images in Fig. S11 (supplementary information), applying a magnetic field to CT-MFBP induces ROS generation, leading to the cleavage of TK-COOH and, subsequently, the time-dependent decomposition of the nanoreactor, characterized by the separation of MnFe_2O_4 -PEG and BP. The potential AMF-triggered TK bond breakage and structural decomposition mechanism is illustrated in Fig. S12 (supplementary information).

3.3 Biocompatibility and antitumor effect of L-MFBP in vitro

The nanoreactor's biosafety at the cellular level was initially investigated. As shown in Figs. 3a and 3b, cell viability in

both SV-HUC-1 cells and human ECs remained above 90% for T-MFBP, D-MFBP, and L-MFBP, indicating favorable biocompatibility over a broad dosage range in these materials. The antitumor effect of these nanomaterials under different conditions was also assessed. The CCK-8 assay demonstrated concentration-dependent cytotoxicity for all materials. The L-MFBP group displayed the highest inherent cytotoxicity without stimulation. The sequential AMF+Las treatment significantly potentiated the antitumor efficacy in all groups. Notably, the L-MFBP+AMF+Las group had the lowest cell viability (16.5%), which was significantly lower than that of all other groups (Fig. 3c). As shown in Fig. S13 (supplementary information), the results demonstrate that the ROS scavenger NAC pretreatment mitigates cytotoxicity by approximately 48% and 35% in the L-MFBP+AMF+Las group and the D-MFBP+AMF+Las group, respectively. The prominent reversion of the cytotoxic effect confirms the ROS dependence of the antitumor activity for the chiral MFBP nanoreactor.

To visually corroborate the cytotoxicity results, live/dead cell staining was conducted using Calcein-AM and BOBO-3. The L-MFBP+AMF+Las group showed predominantly red fluorescence, with nearly absent green signal, indicating extensive cell death compared with other groups. Semi-quantitative analysis further confirmed that L-MFBP+AMF+Las induced the highest cell death rate under stimuli (Figs. 3d and 3e). Intracellular ROS generation, detected by DCFH-DA, was further investigated. Sequential AMF+Las treatment potentiated ROS production most effectively in the L-MFBP+AMF+Las group (Fig. 3f). Semi-quantification (Fig. 3g) revealed that ROS generation in the L-MFBP+AMF+Las group was 5.6 times that in the L-MFBP group, demonstrating the stimulation's powerful enhancing effect. More importantly, the ROS level in the L-MFBP group was also 2.43 times that of the D-MFBP group under AMF and laser stimuli, revealing a pronounced chiral effect that governed the stimulated ROS yield. Subsequently, JC-1 staining was employed to assess mitochondrial membrane potential (Fig. 3h). Consistent with previously documented elevated ROS levels, the L-MFBP group exhibited the most pronounced mitochondrial membrane damage, characterized by a marked increase in green fluorescence and a concurrent decrease in red fluorescence. Semi-quantitative analysis (Fig. 3i) confirmed that the profound ROS accumulation induced by the sequential stimulation of L-MFBP effectively disrupted the mitochondrial membrane potential, thereby committing cells to the apoptotic pathway.

The sequential events of ROS burst and mitochondrial dysfunction thus led to the induction of apoptosis. Quantification by Annexin V-FITC/PI flow cytometry showed that the L-MFBP+AMF+Las group exhibited the highest apoptosis rate (approximately 61%), significantly surpassing the other groups. This solidifies the complete mechanistic pathway

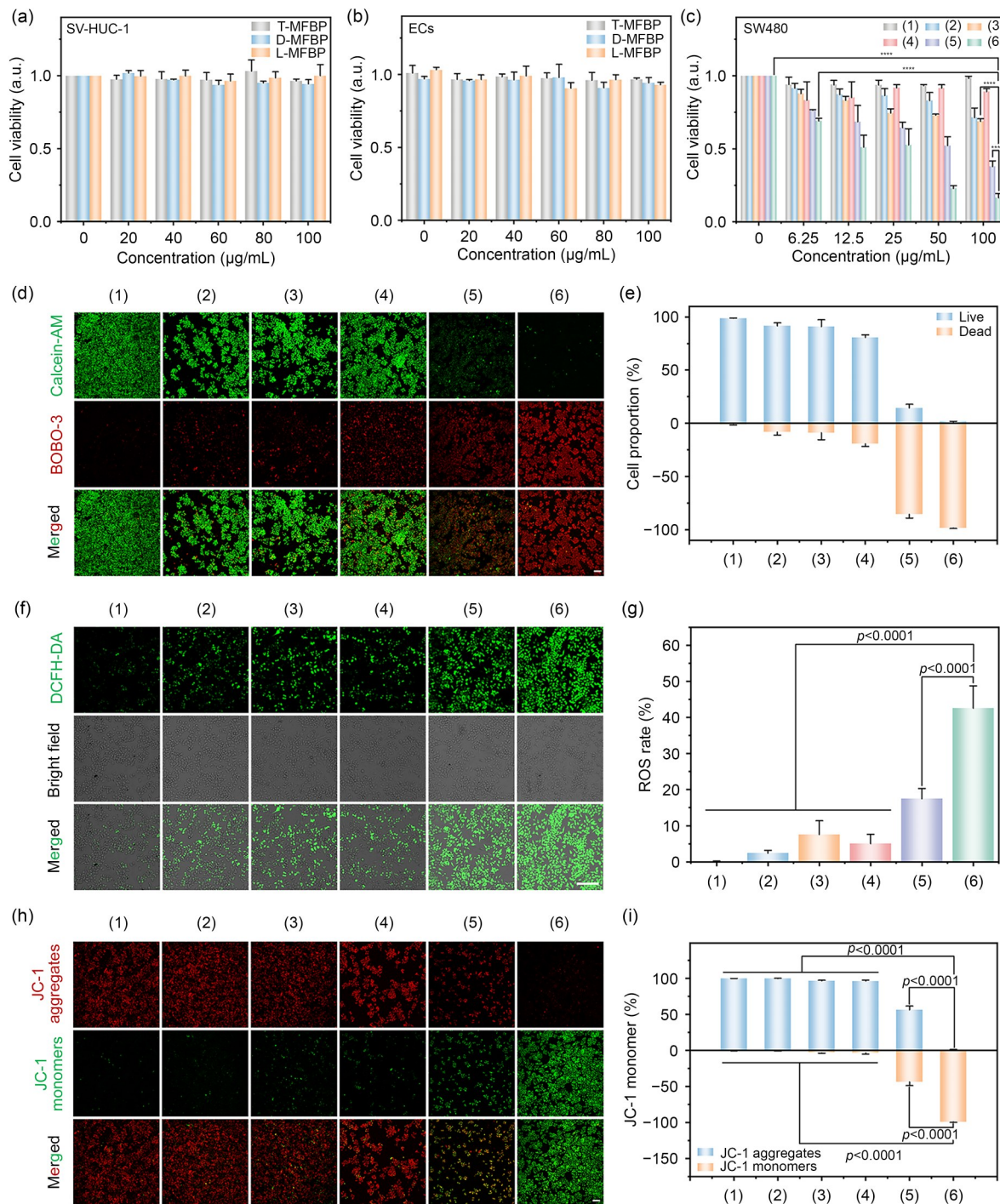


Fig. 3 Assessment of the biocompatibility and antitumor effect in vitro. (a–c) In vitro cytotoxicity of T-MFBP, D-MFBP, and L-MFBP at various concentrations against SV-HUC-1 cells (a), endothelial cells (ECs) (b), and SW480 cells (c), as determined by the cell counting kit-8 (CCK-8) assay. Cell viability is presented relative to the control group. (d) Representative live/dead staining (calcein acetoxymethyl ester (Calcein-AM)/BOBO-3) images of SW480 cells after treatment under different conditions: (1) T-MFBP; (2) D-MFBP; (3) L-MFBP; (4) T-MFBP+AMF+Las; (5) D-MFBP+AMF+Las; (6) L-MFBP+AMF+Las. Green fluorescence indicates live cells; red fluorescence indicates dead cells. Scale bar: 100 nm. (e) Semi-quantification of live/dead staining results based on the fluorescence intensities from (d). Data are presented as the ratio of dead to live cells. (f) Representative fluorescence images of intracellular reactive oxygen species (ROS) generation in SW480 cells under the same treatment conditions as in (d), stained with 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). Scale bar: 100 nm. (g) Semi-quantification of intracellular ROS levels based on the fluorescence intensities from (f). (h) Representative fluorescence images of mitochondrial membrane potential (MMP) in SW480 cells under the same treatment conditions, stained with 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1). Scale bar: 100 nm. (i) Semi-quantification of the MMP loss based on the JC-1 fluorescence ratio (green/red) from (h). Data are expressed as mean±standard deviation ($n=3$). Statistical significances are indicated as *** $p<0.001$ and **** $p<0.0001$

from AMF+Las stimulation to catalyzing ROS production, accumulating oxidative stress, promoting mitochondrial

damage, and finally triggering apoptotic cell death. (Figs. 4a and 4b). To further elucidate the apoptosis mechanism, we

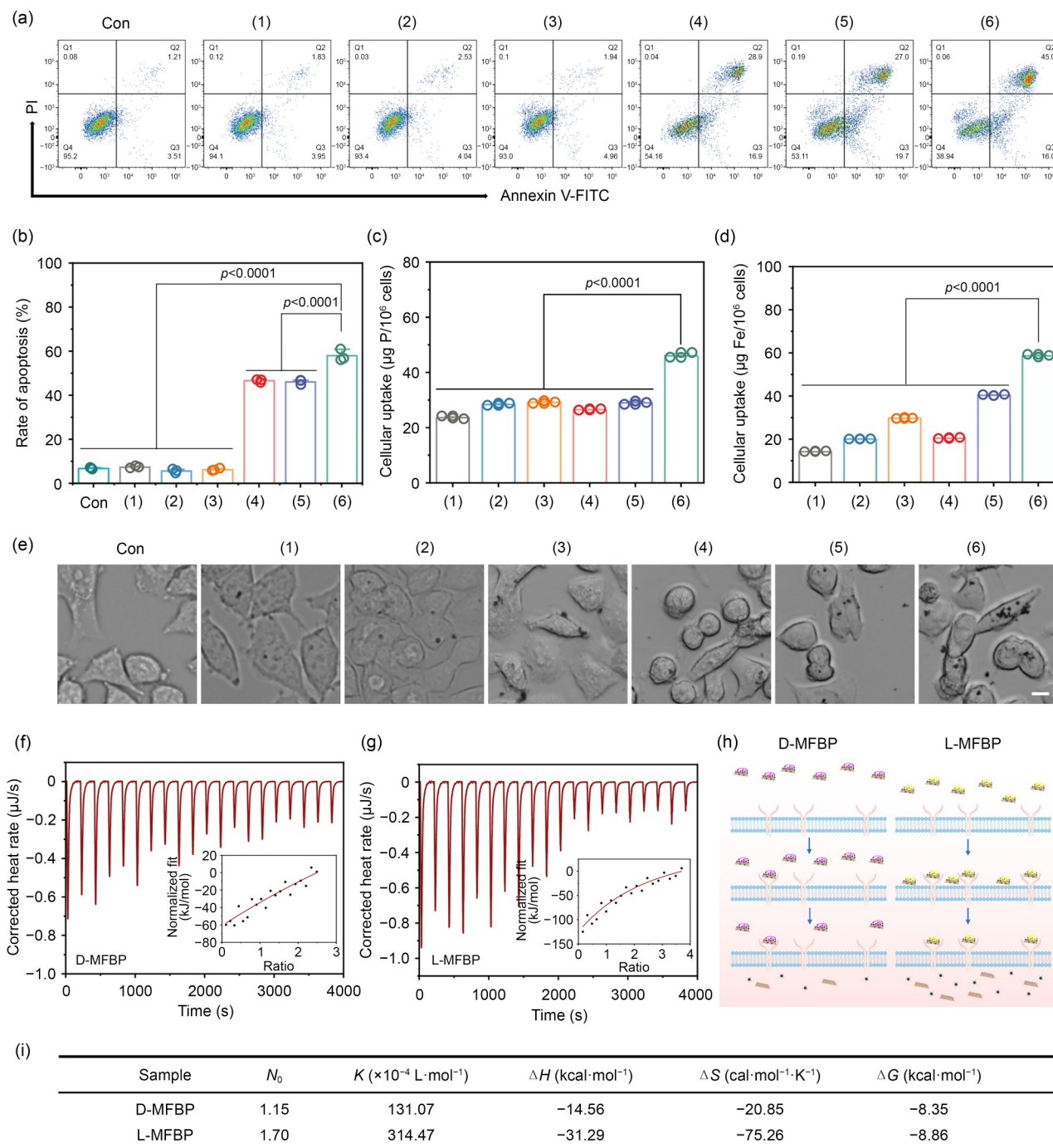


Fig. 4 Investigation of chirality-dependent cellular uptake. (a) Flow cytometry analysis of anoikis in SW480 cells treated under the specified conditions. (b) Quantitative analysis of the anoikis cell population based on flow cytometry data in (a). (c, d) Intracellular content of phosphorus (P) (c) and iron (Fe) (d) in SW480 cells after incubation with different materials. (e) Confocal microscopy images showing the cellular uptake of nanoreactors by SW480 cells. Scale bar: 100 nm. (f, g) Representative raw isothermal titration calorimetry (ITC) thermograms obtained during the titration of D-chiral nanomaterial (f) and L-chiral nanomaterial (g) into a suspension of cell membrane vesicles. Each peak corresponds to the heat change from a single injection. (h) Mechanism diagram of the affinity between the cell membrane and the chiral nanoreactor. (i) Specific parameters of ITC. The groups were assigned as Con (control group), (1) (T-MFBP), (2) (D-MFBP), (3) (L-MFBP), (4) (T-MFBP+AMF+Las), (5) (D-MFBP+AMF+Las), and (6) (L-MFBP+AMF+Las). N_0 : stoichiometry; K : binding constant; ΔH : enthalpy change; ΔS : entropy change; ΔG : Gibbs free energy change. Data in (b–d) are presented as mean±standard deviation ($n=3$)

investigated the potential chirality-dependent cellular uptake efficiency. The intracellular Fe content and P content were quantified using ICP spectrometry, while cell uptake was visualized using confocal microscopy. As shown in Fig. 4c, the P content in the L-MFBP+AMF+Las group was the highest, being 1.65-fold greater than that in the L-MFBP and D-MFBP+AMF+Las groups. Concurrently, the Fe content discrepancy was consistent with that of P content, measuring 2.0-fold and 1.48-fold higher than in the L-MFBP and D-MFBP+AMF+Las groups, respectively (Fig. 4d). These results indicate that both the sequential AMF+Las stimulation and the chiral configuration play significant roles in promoting cellular uptake. Consistent with the elemental assays, direct observation by confocal microscopy (Fig. 4e) further confirmed the highest cellular uptake in the L-MFBP+AMF+Las group, as observed in the ICP analysis.

To characterize the thermodynamic interaction between the cell membrane and the nanoreactor, ITC was further examined at the molecular level (Figs. 4f and 4g). As shown in Fig. 4i, both types of nanoreactors exhibited spontaneous binding with favorable enthalpy changes, and the affinity between the membrane fragments and L-MFBP was approximately 2.4-fold stronger than that with D-MFBP in terms of K_d ($K_d=1/K$, K is a binding constant). Specifically, the K_d value for L-MFBP was 3.18×10^{-7} mol/L, corresponding to an association constant $K \approx 3.14 \times 10^6$ L·mol⁻¹, whereas D-MFBP showed a K_d of 7.63×10^{-7} mol/L ($K \approx 1.31 \times 10^6$ L·mol⁻¹). This confirms the significantly stronger binding affinity of L-MFBP to the SW480 cell membrane. Furthermore, the binding was driven by a favorable enthalpy change ($\Delta H = -130.91$ kJ·mol⁻¹ for L-MFBP and -60.89 kJ·mol⁻¹ for D-MFBP), accompanied by a loss in entropy ($\Delta S = -314.9$ J·mol⁻¹·K⁻¹ and -87.21 J·mol⁻¹·K⁻¹, respectively), indicating that the interaction is enthalpy-dominated and involves substantial molecular ordering upon binding. Thus, the efficient uptake of L-MFBP by tumor cells may result from its strong affinity for the tumor cell membrane (Fig. 4h). The above results indicate that, through chirality-dependent cell membrane binding and uptake, and magnetic-laser-triggered ROS accumulation, CT-MFBP successfully initiates apoptosis and induces tumor cell death.

3.4 In vivo antitumor effect and anoikis induction of L-MFBP

The in vivo therapeutic performance and the outcome of anoikis induction were evaluated in a SW480 subcutaneous tumor model. After the tumor-bearing mouse model had been established, the treatment schedule (Fig. 5a) involved an intratumoral injection on Day 8, followed by sequential AMF-laser stimulation. The treatment was reapplied during subsequent booster treatments on Day 12, Day 16, and Day 20. Tumor volume was recorded every 2 d, and all mice were

euthanized on Day 22 for final endpoint analysis. As shown in Fig. 5b, all groups of mice exhibited a steady increase in body weight over the 22-day treatment period, suggesting no significant systemic toxicity from the treatments. The tumor growth curves in Fig. 5c and the corresponding excised tumor weights in Fig. 5d reveal that L-MFBP+AMF+Las exhibited the most potent tumor-suppressing capability. Specifically, the average tumor weight in the L-MFBP+AMF+Las group was approximately 0.6497 g, compared with 2.082 g in the control group, corresponding to an inhibition rate of 68.79%. Representative photographs of the resected tumors (Fig. 5e) provided visual confirmation of this superior efficacy.

Fluorescence imaging after administration demonstrates the ROS-responsive disassembly of the nanoreactor upon AMF+Las stimulation (Fig. 5f). The high initial fluorescence at 0 min indicates the intact structure. The subsequent signal decrease from 10 to 120 min reflects TK linker cleavage triggered by ROS generated during AMF+Las treatment, leading to the gradual Cy7 release and clearance. This signal attenuation provides direct visual evidence of substantial ROS generation at the tumor site, confirming magnetic-laser-triggered ROS accumulation and multi-step therapy in vivo. Furthermore, histopathological analysis of major organs (Fig. 5g) showed no obvious lesions across all groups, indicating the high biocompatibility and biosafety of these agents. The long-term metabolic fate of the MnFe₂O₄ component can be elucidated in a manner similar to that of other iron-based nanomaterials. After these particles enter systemic circulation, they are predominantly absorbed by the mononuclear phagocyte system, particularly Kupffer cells in the liver and macrophages in the spleen [52, 53]. In the acidic, enzymatic environment of lysosomes, MnFe₂O₄ gradually degrades, releasing free iron and manganese ions that can either be reintegrated into endogenous iron pools and metal-chelating proteins, such as ferritin and hemoglobin, or excreted from the body, thereby mitigating long-term accumulation and toxicity [54, 55]. Additionally, staining of the tumor slice demonstrated that the L-MFBP+AMF+Las group exerted a potent cytotoxic effect and caused the most severe damage to the tumor. Notably, intratumoral delivery enables high local tumor accumulation of the nanoreactor, minimizes systemic exposure, and facilitates precise spatiotemporal control over the magnetic-laser stimulation for subsequent satisfactory therapy. Additionally, effective induction of anoikis within the primary tumor is expected to reduce the source of circulating tumor cells and micrometastases, thereby indirectly contributing to the suppression of tumor dissemination.

The potent in vivo anoikis-inducing mechanism was subsequently analyzed (Fig. 6a). The Western blot results demonstrated a concerted activation of the p38 MAPK pathway

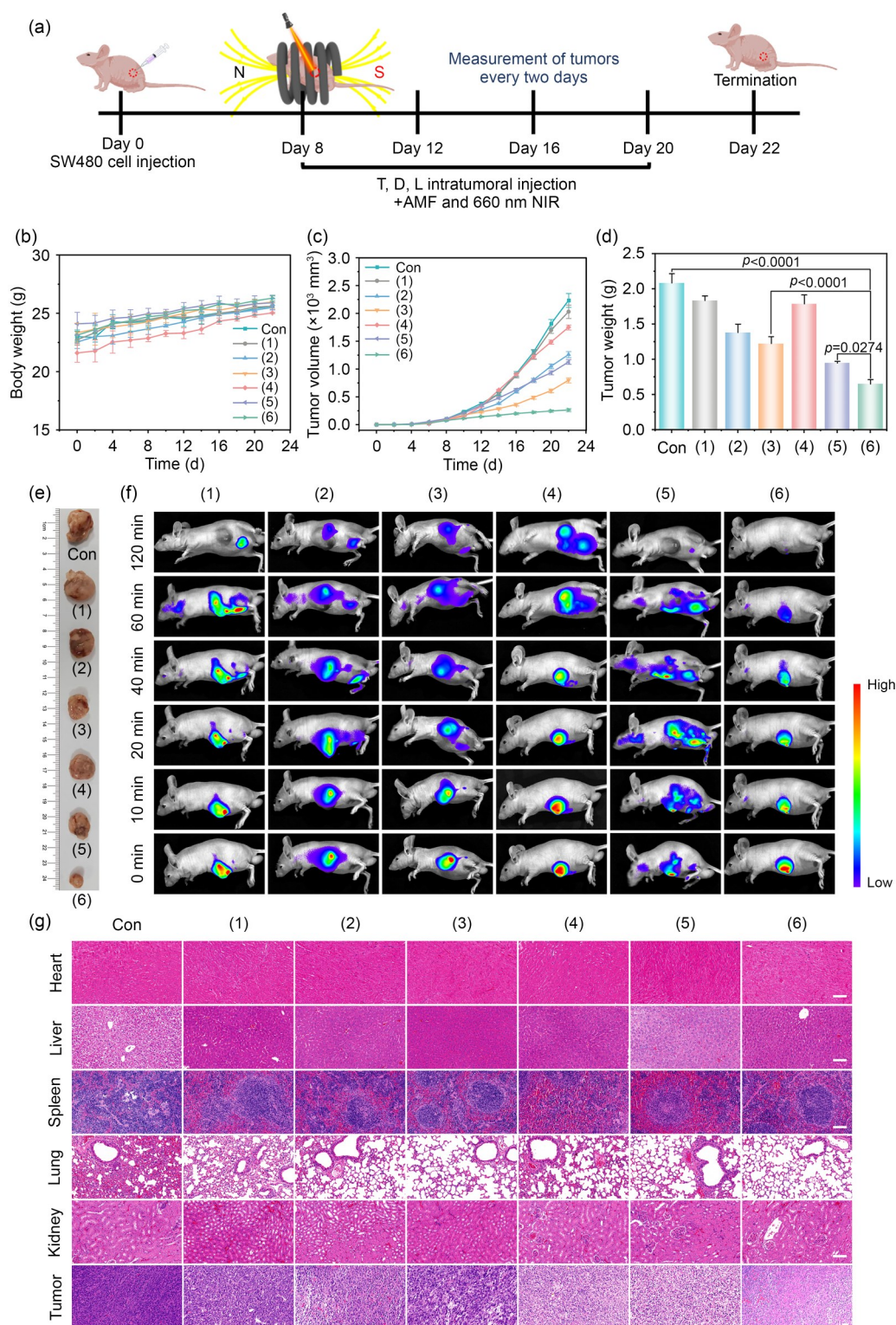


Fig. 5 Antitumor efficacy and biocompatibility assessment in a subcutaneous SW480 tumor model. (a) Schematic illustration of the therapeutic regimen for mice bearing subcutaneous SW480 tumors treated with L-MFBP. (b) Body weight changes of mice in different treatment groups throughout the experimental period. (c) Tumor volume growth curves of mice in different treatment groups. (d) Weight of excised tumors from each group at the endpoint of the study. (e) Representative photographs of excised tumors from each group. (f) In vivo fluorescence imaging of mice after intratumoral injection of the indicated materials, showing distribution and retention at the tumor site. (g) Representative hematoxylin and eosin (H&E)-stained sections of major organs (heart, liver, spleen, lung, kidney, and tumor) collected from mice post-treatment, demonstrating the biosafety of the materials. Scale bars: 100 μ m. The groups were assigned as Con (control group), (1) (T-MFBP), (2) (D-MFBP), (3) (L-MFBP), (4) (T-MFBP+AMF+Las), (5) (D-MFBP+AMF+Las), and (6) (L-MFBP+AMF+Las). Data are expressed as mean $\pm$ standard deviation ($n=3$)

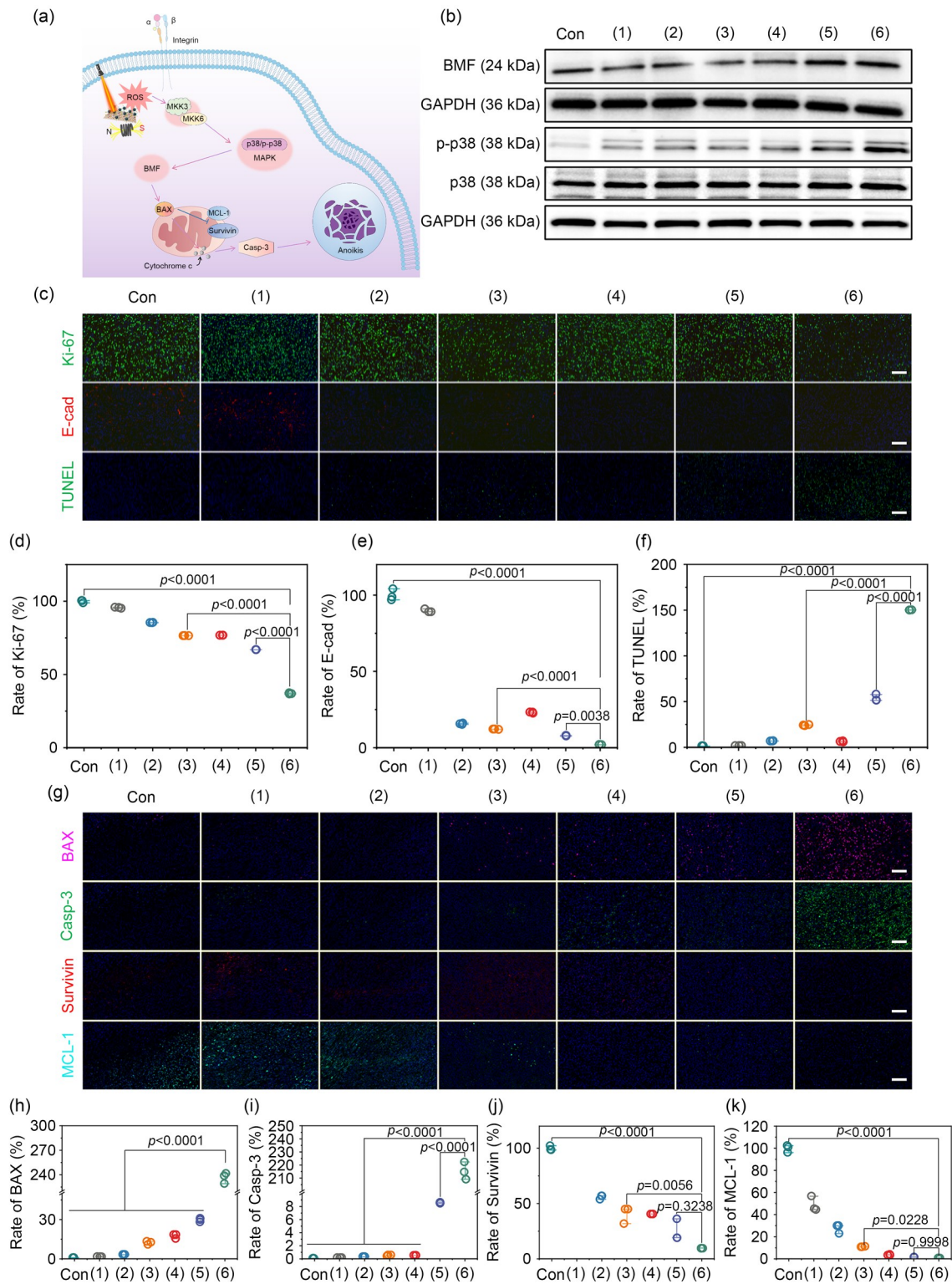


Fig. 6 Investigation of anoikis mechanisms in vivo. (a) Schematic illustration of the anoikis process triggered by the nanoreactor under AMF+Las stimulation. (b) Western blot analysis of key proteins in the p38 MAPK signaling pathway in tumor tissues from the different treatment groups. (c) Representative immunofluorescence staining of tumor sections for E-cadherin (E-cad, red), Ki-67 (green), and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL, green). Blue: 4',6-diamidino-2-phenylindole (DAPI). (d–f) Semi-quantitative analysis of Ki-67-positive cells (d), E-cad fluorescence intensity (e), and TUNEL-positive cells (f). (g) Representative immunofluorescence staining of tumor sections for BAX, Casp-3, Survivin, and MCL-1. Blue: DAPI. (h–k) Semi-quantitative analysis of the fluorescence intensity for BAX (h), Casp-3 (i), Survivin (j), and MCL-1 (k). Scale bars: 100 μm. The groups were assigned as Con (control group), (1) (T-MFBP), (2) (D-MFBP), (3) (L-MFBP), (4) (T-MFBP+AMF+Las), (5) (D-MFBP+AMF+Las), and (6) (L-MFBP+AMF+Las). Data are expressed as mean±standard deviation ($n=3$)

in the L-MFBP+AMF+Las group, evidenced by a notable upregulation of the pro-apoptotic protein BMF and a significant increase in the p-p38/p38 ratio (Fig. 6b). Specifically, compared with the control group, the L-MFBP+AMF+Las group exhibited a 7.82-fold increase in p-p38/p38 (Fig. S14a in the supplementary information) and a 1.85-fold upregulation of the pro-apoptotic effector BMF (Fig. S14b in the supplementary information). Consistently, TUNEL staining revealed the most intense apoptotic signal in the L-MFBP+AMF+Las group (Fig. 6c). The semi-quantification results for the L-MFBP+AMF+Las group increased by 159% compared with those of the D-MFBP+AMF+Las group (Fig. 6f), thereby validating the potent pro-apoptotic outcome of the initiated signaling cascade. Furthermore, immunofluorescence analysis demonstrated concomitant suppression of tumor proliferation and malignancy, characterized by a decrease in the Ki-67-positive rate to 37% (Figs. 6c and 6d). Upon the initiation of p38 MAPK, the activation of Casp-3 cleaves mitochondrial components, thereby inducing anoikis. This process results in a significant reduction in E-cadherin expression, which was lowest in the L-MFBP+AMF+Las group, reaching approximately 2% compared with the control group (Figs. 6c and 6e). Based on the above results, the anoikis induction mechanism can be elucidated as follows: upon magnetic-laser stimulation, the accumulation of ROS initiates a downstream cascade by activating MKK3 or MKK6. These kinases subsequently phosphorylate and activate p38 MAPK, which induces expression of the pro-apoptotic effector BMF. BMF triggers mitochondrial dysregulation by activating BAX and inhibiting Bcl-2, Bcl-6, and Survivin, leading to cytochrome c release, Casp-3 activation, and final execution of anoikis.

To further delineate the molecular events underlying anoikis, immunofluorescence analysis was performed to assess key regulators of mitochondrial apoptosis. As illustrated in Fig. 6g, the L-MFBP+AMF+Las group demonstrated the highest fluorescence intensity for the pro-apoptotic effector BAX and the executioner protease Casp-3, alongside the lowest signals for the anti-apoptotic regulators MCL-1 and Survivin. Semi-quantitative measurements revealed that the BAX expression level in the L-MFBP+AMF+Las group was 7.7 times greater than that in the D-MFBP+AMF+Las group (Fig. 6h), while the Casp-3 expression level was 24.97 times higher (Fig. 6i). Survivin and MCL-1 expression decreased by 15.2% and 0.88% (Figs. 6j and 6k), respectively. This coordinated profile, which features BAX-driven mitochondrial pore formation and Casp-3-mediated proteolytic cleavage, coupled with suppression of key survival proteins, strongly supports the activation of a mitochondrial-dependent apoptotic pathway under AMF+Las stimulation. Together with previous evidence of ROS/p38 MAPK/BMF activation, these results establish a coherent signaling network through which the nanoreactor induces chirality-enhanced anoikis *in vivo* when exposed to a magnetic field and a laser.

3.5 Antitumor mechanism of L-MFBP by transcription analysis

We investigated the molecular mechanisms underlying the tumor-suppressive effects of a chiral nanoreactor using bioinformatics analysis, focusing on its ability to modulate tumor cell death pathways. Gene expression analysis using volcano plots revealed significant upregulation and downregulation of genes in the L-MFBP+AMF+Las group compared with the control group (Fig. 7a). These changes were consistent with the treatment's ability to induce oxidative stress and apoptosis, further supporting the hypothesis that the L-MFBP+AMF+Las group promotes anoikis by enhancing tumor cell detachment from the ECM. KEGG pathway analysis (Fig. 7b) identified alterations in key pathways related to cell adhesion, cytoskeletal regulation, and cell signaling, which are central to tumor cell death. Notably, the downregulation of ECM and cell adhesion-related pathways suggests that L-MFBP+AMF+Las facilitates tumor cell detachment, leading to anoikis. GO (Fig. 7c) analysis further highlights the enrichment of genes involved in oxidative stress, cellular movement, and apoptosis. These processes are essential for the tumor-suppressive effect, as oxidative stress plays a crucial role in anoikis. The PPI network (Fig. 7d) analysis revealed that genes associated with regulation of the actin cytoskeleton and oxidative stress were significantly upregulated in the L-MFBP+AMF+Las group, further supporting the anoikis mechanism. Gene heatmap (Fig. 7e) analysis revealed pronounced changes in the expression of genes associated with oxidative stress and apoptosis in the L-MFBP+AMF+Las group, which aligns with the enhanced tumor cell death observed in this group. The combination of a chiral nanoreactor with AMF+Las proved to be the most effective in inducing tumor cell death via anoikis, thereby offering a promising strategy to overcome tumor resistance mechanisms. In summary, transcriptomic profiling demonstrates that anti-tumor efficacy is intrinsically linked to the nanoreactor's capacity to disrupt ECM adhesion and cytoskeletal integrity. The sequential AMF+Las stimulation significantly amplifies this effect, with the enhancement being most pronounced for the L-MFBP. These findings provide a robust molecular rationale for the observed phenotypic outcomes and underscore the promise of combining a chiral nanoreactor with magnetic-laser stimuli-responsive properties for cancer therapy via the anoikis induction pathway. Additionally, transcriptomic analysis revealed the upregulation of damage-associated molecular patterns (DAMPs), including heat shock protein 70 (HSP70) and HSP90 (Fig. 7c). Meanwhile, KEGG pathway enrichment analysis identified several pathways related to pathogen infection (Fig. 7b). These results suggest that the treatment may induce immune- and stress-related cellular responses and potentially influence antigen processing and presentation. However, further experimental validation, such as immune cell

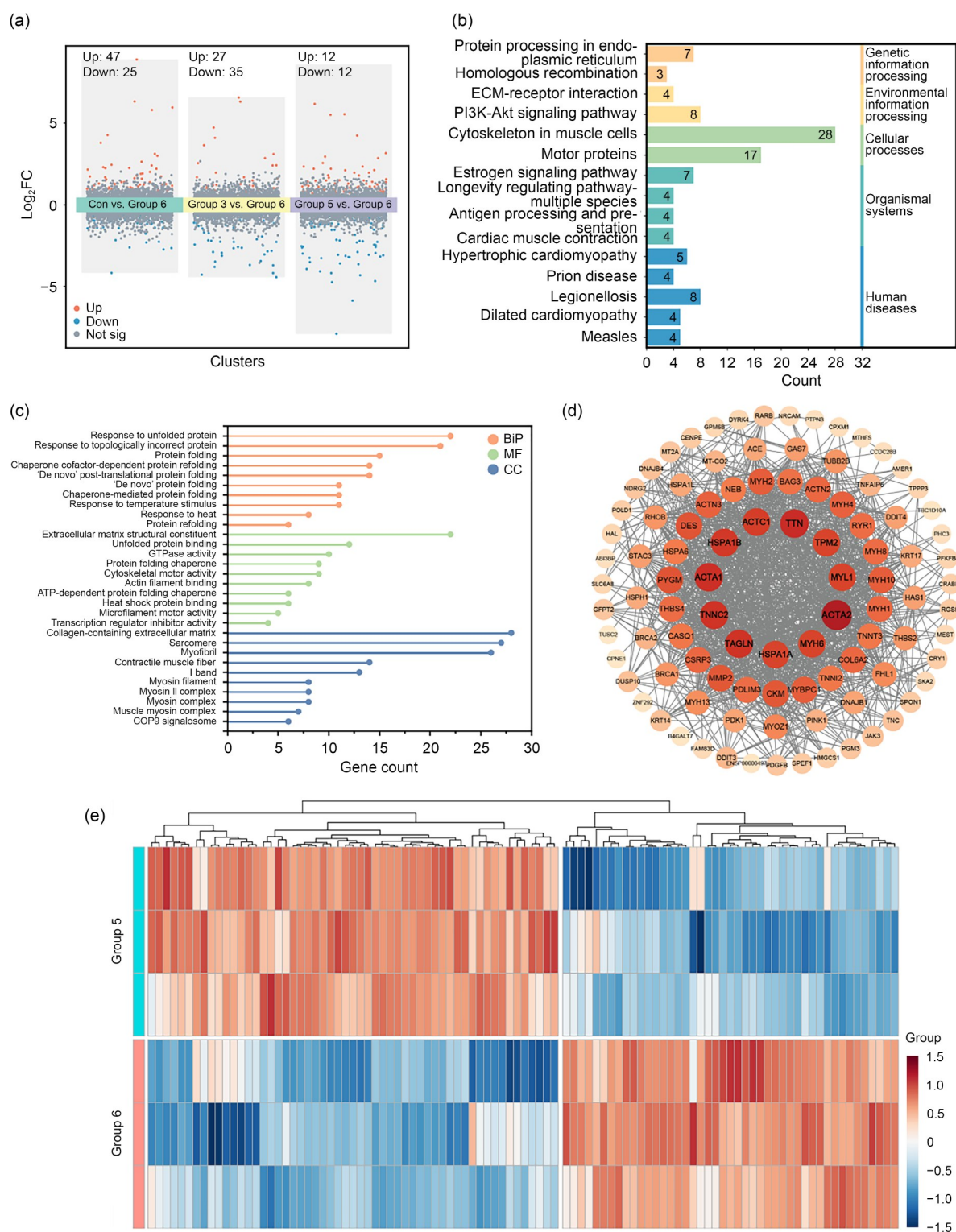


Fig. 7 Transcriptome analysis of tumors post-treatment. (a) Volcano plots displaying differentially expressed genes (DEGs) from the comparisons: Group 6 vs. Con, Group 6 vs. Group 3, and Group 6 vs. Group 5. Upregulated genes are shown in orange, downregulated genes in blue, and nonsignificant genes in gray. (b) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis for DEGs identified in the Group 6 vs. Group 5 comparison. (c) Gene Ontology (GO) enrichment analysis (biological process (BiP), cellular component (CC), and molecular function (MF)) for DEGs from the Group 6 vs. Group 5 comparison. (d) Protein–protein interaction (PPI) network of key proteins encoded by DEGs from the Group 6 vs. Group 5 comparison, generated using the STRING database. (e) Heatmap depicting the expression patterns of selected DEGs from the Group 6 vs. Group 5 comparison across different treatment groups. The groups were assigned as Con (control group), Group 1 (T-MFBP), Group 2 (D-MFBP), Group 3 (L-MFBP), Group 4 (T-MFBP+AMF+Las), Group 5 (D-MFBP+AMF+Las), and Group 6 (L-MFBP+AMF+Las)

infiltration analysis, is required to confirm whether functional antitumor immune activation is involved.

4 Conclusions

The development of therapies that specifically target anoikis pathways in tumor cells has become a highly promising strategy for combating tumor progression and metastasis. From this perspective, we meticulously engineered a MnFe_2O_4 -BP chiral magnetic nanoreactor and examined its magnetic-laser stimuli-responsive performance, along with its antitumor effects both in vitro and in vivo. This reactor demonstrates enzyme-like activity, generating sufficient ROS and O_2 when exposed to AMF and a laser, thereby facilitating the responsive release of MnFe_2O_4 -PEG and BP and alleviating hypoxic conditions within tumors while promoting PDT. After ROS accumulation, the MAPK signaling pathway is activated, particularly the p38 pathway. This activation subsequently induces the expression of BMF, a pro-apoptotic protein, resulting in mitochondrial damage. The increased expression of pro-apoptotic proteins and the reduced levels of anti-apoptotic proteins ultimately trigger Casp-3 activation, promoting anoikis in tumors. In addition, in vitro and in vivo experiments demonstrate that tumor cells uptake L-MFBP twice as much as D-MFBP, highlighting significant chirality-dependent uptake characteristics, confirmed by material–cell membrane interactions. Furthermore, L-MFBP exhibits superior tumor-inhibition and anoikis-induction effects compared with D-MFBP. Additionally, owing to similarities in microenvironmental features between tumors and pathogenic infections, such as oxidative stress imbalance and hypoxia, as well as chiral interfaces in biological systems, chiral MFBP nanoreactors may hold potential for broader biomedical applications. This study primarily focuses on the design of chiral magnetic-laser-responsive nanoplatfoms and their role in enhancing anoikis to inhibit tumor metastasis, offering insights into the potential of regulating anoikis in cancer therapy.

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Author contributions BC and CML are responsible for designing the research topics, experimental ideas, and research schemes. YW, JW, SCL, and MJL are responsible for the experimental operation and data processing. RCF, XL, CSY, YYC, and ZQM are responsible

for data analysis. YW, CXG, CML, and BC are responsible for drafting and revising the paper.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All animal experiments were performed following the guidelines for the care and use of laboratory animals established by the Affiliated Hospital of Integrated Traditional Chinese and Western Medicine, Nanjing University of Chinese Medicine. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Hospital (Ethical Lot Number: 2022-LWKYS-046).

Data availability The data that support the findings of this study are available from the corresponding authors upon reasonable request.

Use of generative AI tools During the preparation of this manuscript, the authors used DeepSeek-R1 for language refinement and grammar editing to improve readability and clarity. The tool was not used to generate scientific content, research data, figures, or conclusions. All AI-generated outputs were carefully reviewed and edited by the authors as needed. The authors take full responsibility for the integrity, accuracy, and originality of the content of this publication.

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