Nanoscale covalent organic framework for the low-temperature treatment of tumor growth and lung metastasis

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ABSTRACT Photothermal therapy (PTT) has shown promising applications in tumor therapies. However, due to laser-induced nonspecific heating and heat diffusion, high levels of hyperthermia ($>50^{\circ}\text{C}$) in tumor tissues often increase the risk of cancer recurrence and metastasis, which causes the patient pain and destroys the surrounding normal cells and tissues. It is therefore important to develop photothermal strategies that have excellent therapeutic efficiencies under low-temperature conditions ($\leq 45^{\circ}\text{C}$). In addition, the heterogeneity and complexity of tumors require the development of combinatorial antitumor treatments as the therapeutic efficiency of monomodal PTT is not currently sufficient. Herein, we have adopted a stepwise synthetic approach to develop a highly efficient multimodal therapeutic agent GA@PCOF@PDA by successive bonding defect functionalization (BDF), guest encapsulation, and surface modification steps. The covalently grafted porphyrinic photosensitizers (Por), encapsulated gambogic acid (GA), and surface-modified PDA film are independently responsible for photodynamic therapy (PDT), heat-shock protein 90 (HSP90) down-regulation and chemotherapy (CT), and low-temperature PTT. This proof-of-concept study illustrates an efficient, generalized approach to design high-performance covalent organic framework (COF)-based nanoagents that can be easily tailored to combine different therapeutic modalities for improved cancer theranostics at low temperatures.

Keywords: covalent organic framework, low-temperature photothermal therapy, gambogic acid, heat-shock protein 90, lung metastasis

INTRODUCTION

Photothermal therapy (PTT) has received a large amount of attention, as it is a non-invasive operation and has tunable phototoxicity and low systemic toxicity [1]. Typically, a photothermal agent can utilize near-infrared (NIR) light absorption to generate local hyperpyrexia to damage tumor cells [2]. For effective tumor ablation, the temperature in the tumor region usually requires hyperthermia of 50°C [3]. However, owing to laser-induced nonspecific heating and heat diffusion, the high levels of hyperthermia in tumor tissues often increase the risk of cancer recurrence and metastasis, which causes large amounts of pain for the patient, and destroys the surrounding normal cells

and tissues [4–6]. Therefore, the development of more feasible photothermal strategies with excellent therapeutic efficiencies under low-temperature conditions ($\leq 45^{\circ}\text{C}$) would be of significant value for the future clinical translation of PTT.

Low-temperature photothermal therapeutic effects, however, often suffer due to the barrier of the heat shock proteins (HSPs). Cells and tissues generally secrete HSPs to protect themselves from thermal damage when the tumor temperature is slightly heated to $\sim 41^{\circ}\text{C}$. Furthermore, cancer cells have a self-repair ability and can recover from the lesion damage caused by higher temperatures (e.g., 43°C) with the help of HSPs [7]. Hence, increasing the thermal sensitivity of tumor cells by inhibiting the synthesis of HSPs is a potential method to improve the efficiency of low-temperature heating treatments [8,9]. In addition, the heterogeneity and complexity of tumors often limits the therapeutic efficiency of PTT [10–11]. Recently, PTT-based combination therapy, especially for combined photodynamic therapy (PDT) and PTT, has drawn wide attention [12–14]. Since monomodal low-temperature PTT usually cannot improve the treatment efficacy, especially for the simultaneous tumor growth inhibition and lung metastasis, a low-temperature PTT-involved multimodal therapy might be a suitable alternative, although it has not yet been reported.

As a new emerging class of porous materials, covalent organic frameworks (COFs) have attracted tremendous attention in the field of biomedicine due to their well-defined skeletons, large surface areas, structural/chemical diversities, and multifunctionalities [15–17]. We and others have recently developed a series of nanoscale COF (NCOF)-based nanomedicines for chemotherapy (CT) [18–20], phototherapy (PDT [21] and PTT [22,23]), and Ca^{2+} -overlaid therapy for the treatment of different cancers [24]. As a continuation of the COF-based antitumor study, we report herein an NCOF-based multifunctional nanoagent of GA@PCOF@PDA which consists of a porphyrin (Por)-decorated COF, gambogic acid (GA), and polydopamine (PDA). It was prepared by consecutive bonding defect functionalization (BDF) [25], guest encapsulation, and surface modification steps [26,27], such that the Por-decorated porous COF framework was filled with GA molecules and its exterior coated with a PDA membrane (Scheme 1). Overall, GA@PCOF@PDA demonstrates the following major advantages, which are also the rationale behind the design: (a) logical integration of simple, nontoxic functional components to generate a multifunctional, high-performance yet highly biocompatible nanoagent;

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(b) multimodal therapy based on each task-specific species with complementary advantages, such as Por-derived PDT, GA-based heat-shock protein 90 (HSP90) inhibition [28,29] and CT, PDA-dependent low-temperature PTT, and enhanced biocompatibility [30], in a single nanoscale COF platform; and (c) nanocrystallization of functional organic molecules *via* metal-free NCOF-carriers that can not only overcome their poor water solubility, but also avoid the potential toxicity caused by metallo-nanoagents. As expected, GA@PCOF@PDA realizes a remarkably combinatorial low-temperature PTT, CT, and PDT, leading to the strong inhibition of primary tumor progression and cancer metastasis as was demonstrated both *in vitro* and *in vivo* in this investigation.

EXPERIMENTAL SECTION

Materials

All reagents used in this investigation were analytically pure and used directly without further purification. 1,4-Dioxane, glacial acetic acid, and acetonitrile were purchased from Sinopharm Chemical Reagent Co., Ltd. 1,3,5-Tris(4-aminophenyl)benzene, 2,5-dimethoxyterephthalaldehyde, and 5,10,15,20-tetra(4-aminophenyl)porphyrin were all purchased from Jilin Yanshen Technology Co., Ltd. Polyvinylpyrrolidone ($M_w = 8000$), benzaldehyde, and dopamine hydrochloride were purchased from Aladdin Reagent Co., Ltd. (Shanghai). GA was purchased from Yien Chemical Technology Co., Ltd. (Shanghai). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2-*H*-tetrazolium bromide (MTT) was purchased from Sigma-Aldrich (Shanghai) Trading Co. Ltd. A living dead cell staining kit (calcein acetoxymethyl ester (calcein-AM), propidium iodide (PI)) was purchased from Shanghai Yisheng Biotechnology Co., Ltd. 4T1 cells were purchased from Procell Life Science & Technology Co., Ltd. Cell counting kit-8 was purchased from MedChemExpress (MCE). 4% Formaldehyde universal tissue fixative was purchased from Biosharp Co., Ltd. Dulbecco's phosphate-buffered saline (DPBS), and fetal bovine serum (FBS) were purchased from Biological Industries USA. RPMI Medium Modified (1640), penicillin streptomycin mixtures (Pen-Strep), and trypsin-EDTA (ethylene diamine tetraacetic acid) solution (0.25%) were purchased from HyClone Laboratories, Inc.

Instrumentations

Fourier transform infrared (FT-IR) spectra were obtained in the 4000–400 cm^{-1} range using a Thermo Scientific Nicolet iS50 FT-IR spectrometer equipped with a diamond attenuated total reflection (ATR) module. Each spectrum was the average of 16 scans. Ultraviolet-visible (UV-vis) absorption spectra were recorded on a Shimadzu UV-2700 Double Beam UV-vis Spectrophotometer. The morphologies of all the samples were acquired on a Hitachi SU8010 scanning electron microscope (SEM) and a Hitachi HT7700 120kV Compact-Digital transmission electron microscope (TEM). Powder X-ray diffraction (PXRD) patterns were obtained on a Rigaku SmartLab SE X-ray powder diffractometer with Cu K α line focused radiation ($\lambda = 1.5405 \text{ \AA}$) from $2\theta = 2.00^\circ$ up to 50.00° with 0.01° increments. Nitrogen adsorption isotherms were measured at 77 K with a Micromeritics ASAP2020 HD88 surface area and porosity analyser. Hydrodynamic particle sizes and zeta potentials were measured using the Malvern Zetasizer Nano ZS90 System. Laser scanning confocal fluorescence images were captured with a

Leica TCS SP8 confocal laser scanning microscope with an objective lens ($\times 20$). Cell proliferation data were obtained using a full-wavelength, multi-channel microporous plate detector-SpectraMax 190 optical absorption marker.

Synthesis of TPB-DMTP-COF

TPB-DMTP-COF was prepared according to a previously reported method [25]. A mixture solution was first obtained with 1,3,5-tris(4-aminophenyl)benzene (0.492 g, 1.4 mmol), 2,5-dimethoxyterephthalic acid (0.427 g, 2.2 mmol), and polyvinylpyrrolidone ($M_w = 8000$, 1.0 g) dispersed in glacial acetic acid (25 mL) and acetonitrile (500 mL) under stirring at 25°C for 12 h. After the addition of benzaldehyde (20 μL , 0.2 mmol), the aqueous solution was stirred again for 1 h. Then, a yellow powder was obtained by centrifugation, and rinsed with acetonitrile several times.

Synthesis of COF-Por

TPB-DMTP-COF (0.05 g) was mixed with Por (126 mg, 0.2 mmol) in the solution of acetic acid (1.0 mL, 3 mol L^{-1}) and 1,4-dioxane (200 mL). After reflux for 12 h, the solution was centrifuged and washed with 1,4-dioxane until the supernatant was colorless.

Synthesis of GA@PCOF

GA (0.1 mg mL^{-1}) was added into COF-Por (10 mg) with 10 mL of ethanol solution and stirred for 24 h. Then the product was centrifuged at $13,300 \text{ r min}^{-1}$ for 10 min and stored at 4°C until further use.

Synthesis of GA@PCOF@PDA

Dopamine hydrochloride (0.5 mg) was added to GA@PCOF (8.0 mg) with 10 mL of tris buffer (10 mmol L^{-1} , pH 8.5) and stirred at room temperature for 3 h. Then the product was obtained by centrifugation ($13,300 \text{ r min}^{-1}$, 10 min) and dried under vacuum at 30°C .

GA slow-release

GA@PCOF@PDA (1 mg mL^{-1} , 200 μL) was separately added to 800 μL of aqueous solution containing PBS buffer with different pH values (7.4, 6.0, and 5.0). At different time intervals (0, 0.5, 1.5, 2.5, 4, 6, 8, 12 and 24 h) the supernatant that contained GA was obtained by centrifugation ($13,300 \text{ r min}^{-1}$, 10 min). The amount of the released GA was analyzed by measuring the absorbance intensity of the GA at 362 nm.

Singlet oxygen detection

1,3-Diphenylisobenzofuran (DPBF, 10 mmol L^{-1}) was pretreated with TPB-DMTP-COF (160 $\mu\text{g mL}^{-1}$, 200 $\mu\text{g mL}^{-1}$ GA@PCOF@PDA equiv), COF-Por (170 $\mu\text{g mL}^{-1}$, 200 $\mu\text{g mL}^{-1}$ GA@PCOF@PDA equiv), GA@PCOF (180 $\mu\text{g mL}^{-1}$, 200 $\mu\text{g mL}^{-1}$ GA@PCOF@PDA equiv), and GA@PCOF@PDA (200 $\mu\text{g mL}^{-1}$), and then irradiated with 660-nm laser (50 mW cm^{-2}) for 50 s, respectively. The absorbance intensity of the oxidized DPBF was recorded every 10 s at 412 nm.

Photothermal performance evaluation

The photothermal effect of GA@PCOF@PDA (0, 50, 100, 200, 400, and 800 $\mu\text{g mL}^{-1}$) was evaluated by monitoring the temperature variations of the solution under 808-nm laser irradiation (2 W cm^{-2}) for 10 min. The temperature variations of

GA@PCOF@PDA ($200\ \mu\text{g mL}^{-1}$) under 808 nm photoirradiation with various laser power densities ($0.5, 1, 1.5, 2$, and $2.5\ \text{W cm}^{-2}$) were also measured. The photothermal performances of the different samples, such as TPB-DMTP-COF ($160\ \mu\text{g mL}^{-1}$, $200\ \mu\text{g mL}^{-1}$ GA@PCOF@PDA equiv), COF-Por ($170\ \mu\text{g mL}^{-1}$, $200\ \mu\text{g mL}^{-1}$ GA@PCOF@PDA equiv), GA@PCOF ($180\ \mu\text{g mL}^{-1}$, $200\ \mu\text{g mL}^{-1}$ GA@PCOF@PDA equiv), and GA@PCOF@PDA ($200\ \mu\text{g mL}^{-1}$), were recorded by monitoring their temperature variations after 808-nm laser irradiation ($2\ \text{W cm}^{-2}$) for 10 min. To evaluate the photothermal stability of GA@PCOF@PDA, $200\ \mu\text{g mL}^{-1}$ of GA@PCOF@PDA was exposed in the repeated cycle of the 808-nm laser irradiation on/off ($2\ \text{W cm}^{-2}$).

Intracellular singlet oxygen generation

The generation of $^1\text{O}_2$ was detected using a singlet oxygen sensor green (SOSG). The 4T1 cells (1×10^4 cells per well) were seeded into a small confocal dish. After overnight incubation, the adherent cells were separately pre-incubated with COF-Por ($170\ \mu\text{g mL}^{-1}$), GA@PCOF ($180\ \mu\text{g mL}^{-1}$) and GA@PCOF@PDA ($200\ \mu\text{g mL}^{-1}$) and then washed with DPBS carefully. Next, $5\ \mu\text{mol L}^{-1}$ of SOSG was added and incubated with the cells for another 15 min. After being washed with DPBS, the cells were exposed to 660-nm laser irradiation ($50\ \text{mW cm}^{-2}$) for 1 min. Finally, the cells were imaged using a laser confocal microscope (Ex: 488 nm). The same methods mentioned above were used to detect the generation of $^1\text{O}_2$ in the cells treated with GA@PCOF@PDA ($200\ \mu\text{g mL}^{-1}$) with 808-nm laser irradiation ($2\ \text{W cm}^{-2}$).

In vitro hyperthermia experiments

The 4T1 cells were seeded into 96-well plates (1×10^4 cells per well). After overnight incubation, the adherent cells were incubated with the free GA ($5\ \mu\text{g mL}^{-1}$) and GA@PCOF@PDA ($100\ \mu\text{g mL}^{-1}$) for different durations (0, 1, 2, and 4 h) at 37 or 43°C, and the resultant cells were cultured at 37°C for another 24 h. The cell cultured medium was then removed, and $100\ \mu\text{L}$ of MTT solution ($0.5\ \text{mg mL}^{-1}$ in PBS) was added into each well. After 4 h of incubation, the formazan crystals formed in each well were dissolved in $150\ \mu\text{L}$ of dimethyl sulfoxide. Finally, the absorbance intensity was measured at 490 nm with a microplate reader.

In vitro combination therapy effect of GA@PCOF@PDA

The 4T1 cells were seeded into 96-well plates (1×10^4 per well). After overnight incubation, the adherent cells were incubated with different concentrations of GA@PCOF@PDA (0, 25, 50, 100, 150, 200, and $400\ \mu\text{g mL}^{-1}$) for 12 h. After fresh medium was added into each well, the cells were exposed to pointed laser irradiation. For PDT, the cells were irradiated with the 660-nm laser with a power density of $50\ \text{mW cm}^{-2}$ for 5 min. For PTT, the cells were irradiated with 808-nm laser with a power density of $2\ \text{W cm}^{-2}$ for 10 min. For the PDT and PTT, the cells were irradiated with 660-nm laser with a power density of $50\ \text{mW cm}^{-2}$ for 5 min, and then irradiated with 808-nm laser with a power density of $2\ \text{W cm}^{-2}$ for 10 min. Subsequently, the cells were cultured for another 24 h and the MTT assay was employed to detect the cell viability. The experiment for the 4T1 cells stained with calcein-AM/PI was the same as described for the MTT assay. After photoirradiation, calcein-AM ($2\ \mu\text{mol L}^{-1}$) and PI ($4.5\ \mu\text{mol L}^{-1}$) were used to co-stain the live and dead cells for 20 min. The cells were imaged using a laser confocal

microscope (Ex: 488 and 543 nm).

Established in vivo tumor model

All animal studies were performed in accordance with the Animal Care and Use Committee of Shandong Normal University (approval number AECCSDNU2021041). To develop the 4T1 xenograft tumor model, $70\ \mu\text{L}$ of 4T1 cells (1×10^7 , PBS) were subcutaneously injected into the backs of the female BALB/c mice (5 weeks old). The mice were used after the tumors had grown to approximately $100\ \text{mm}^3$. To develop the orthotopic metastatic breast cancer model, $50\ \mu\text{L}$ of 4T1 cells (1×10^6 , PBS) was injected into the third mammary fat pad of the BALB/c nude mice (6–8 weeks old). The mice were used when the tumor volumes were approximately $100\ \text{mm}^3$.

Hemolytic test

Different concentrations of GA@PCOF@PDA (0, 6.25, 12.5, 25, 50, 75, 100, 150, and $200\ \mu\text{g mL}^{-1}$) were incubated with 1 mL of red blood cells suspended in saline. After incubation at 37°C for 4 h, $100\ \mu\text{L}$ of the supernatant was collected by centrifugation and the absorbance intensity at 570 nm was measured using a microplate reader.

For IR thermal imaging

4T1 tumor-bearing mice were injected intravenously with $100\ \mu\text{L}$ of DPBS solution containing GA@PCOF@PDA ($2\ \text{mg mL}^{-1}$). The control mice were intravenously injected with $100\ \mu\text{L}$ of DPBS solution. After 12 h post-injection, the tumor sites were exposed to 808-nm laser ($1.5\ \text{W cm}^{-2}$) for 10 min until their temperature reached 43°C and it was maintained for 10 min. The photothermal images were recorded using an NIR thermal camera.

In vivo antitumor efficiency

When the tumor volume reached about $100\ \text{mm}^3$, 42 tumor-bearing nude mice were randomly divided into seven groups: PBS (i), PBS + 660-nm laser + 808-nm laser (ii), GA@PCOF@PDA (iii), GA@PCOF@PDA + 808-nm laser (iv), GA@PCOF@PDA + 660-nm laser (v), PCOF@PDA + 660- + 808-nm lasers (vi), GA@PCOF@PDA + 660- + 808-nm lasers (vii). The intravenous inject dose of GA@PCOF@PDA was $8\ \text{mg kg}^{-1}$, and that of PCOF@PDA was ($7.6\ \text{mg kg}^{-1}$). Twelve hours after the injection, the light treatment was performed using 808-nm laser ($1.5\ \text{W cm}^{-2}$, 10 min) and/or 660-nm laser ($50\ \text{mW cm}^{-2}$, 5 min). During the experiment, the tumor volume and the weight of the nude mice were recorded every day for 14 days. Additionally, the hematoxylin and eosin (H&E) staining for the paraffin sections of the tumor and organs was in accordance with the manufacturer's protocol.

In vivo inhibition of metastatic cancer

The experimental groups and light treatment procedures were the same as described above. On the 28th day of treatment, the lung tissues of each group were collected, washed with cold PBS, fixed with 4% formalin and photographed. H&E staining of lung was used to access the lung metastasis and record the number of macroscopic metastatic nodules on each lung.

Western blot

For *in vitro* experiments, 4T1 cells were seeded into 6-well plates (5×10^5 per well). After overnight incubation, the adherent cells

were incubated with GA ($5 \mu\text{g mL}^{-1}$), PCOF@PDA ($95 \mu\text{g mL}^{-1}$, $100 \mu\text{g mL}^{-1}$ GA@PCOF@PDA equiv) and GA@PCOF@PDA ($100 \mu\text{g mL}^{-1}$), respectively, in a hyperthermia atmosphere (43°C) for 2 h and further cultured for another 24 h at 37°C . Then cold cell lysis buffer was added to each well and the lysates were collected and centrifuged at 4°C . Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate the cell total protein. After electroblotting onto the poly(vinylidene fluoride) (PVDF) membrane, the membrane was pre-incubated with the primary antibody for 12 h and incubated with the secondary antibody for another one hour. Finally, the HSP90 protein was imaged by a BIORAD ChemiDocTM Touch imaging system.

RESULTS AND DISCUSSION

Synthesis and characterization of GA@PCOF@PDA

A Por-photosensitizer decorated COF-Por, which is a highly efficient singlet oxygen ($^1\text{O}_2$) generator, was first prepared using a nanoscale TPB-DMTP-COF based on our previously published protocols (Scheme 1) [25]. By immersing COF-Por in an EtOH solution of GA at room temperature for 24 h, the host-guest supramolecular system GA@PCOF was readily obtained. As a natural product molecule, GA is not only an effective chemotherapeutic agent, but also a potent inhibitor for 90 kDa HSP (HSP90), which is a key protein that induces the thermo-resis-

tance of cancer cells under hyperthermia [7]. GA@PCOF was then coated with photothermal and biocompatible PDA [30] by mixing it with dopamine in tris buffer (10 mmol L^{-1} , pH 8.5) at room temperature for 3 h, and this resulted in the dark brown GA@PCOF@PDA.

TEM and SEM images show that the obtained COF-Por and GA@PCOF were radial spheres, and they were on average 144 nm, which was similar to the pristine TPB-DMTP-COF [25] (Fig. 1a). The GA@PCOF particles that were tightly coated by PDA had a slight size increase (approximately 180 nm). This observation was further supported by the dynamic light scattering (DLS) measurements (Fig. 1b). The polydispersity index (PDI) measurement of GA@PCOF@PDA was 0.224, indicating its good dispersibility in PBS (pH 7.4) at 25°C . The measured zeta potentials of TPB-DMTP-COF, COF-Por and GA@PCOF were -11.7 ± 0.8 , -13.5 ± 0.5 , and $-15.8 \pm 0.5 \text{ mV}$, respectively, whereas the surface potential of GA@PCOF@PDA sharply decreased to $-24.6 \pm 1.7 \text{ mV}$, due to the surface decoration of the negatively charged PDA (Fig. S1).

The crystalline nature of GA@PCOF and GA@PCOF@PDA was confirmed by their PXRD patterns. Compared with TPB-DMTP-COF ($2\theta = 2.73^\circ, 4.73^\circ, 5.48^\circ, 7.32^\circ, 9.62^\circ$, and 25.26°) and COF-Por ($2\theta = 2.71^\circ, 4.73^\circ, 5.46^\circ, 7.34^\circ, 9.63^\circ$, and 25.37°), no peak shift was observed for GA@PCOF ($2\theta = 2.73^\circ, 4.75^\circ, 5.47^\circ, 7.33^\circ, 9.62^\circ$, and 25.43°) or GA@PCOF@PDA ($2\theta = 2.73^\circ, 4.81^\circ, 5.51^\circ, 7.36^\circ, 9.62^\circ$, and 25.18°) (Fig. 1c), indicating that the

Scheme 1 Schematic illustration of the multifunctional COF-based nanocomposite for low-temperature synergistic tumor therapy. (a) Schematic process of the GA@PCOF@PDA nanocomposite. (b) Schematic illustration of the therapeutic principle.

Figure 1 Characterization of GA@PCOF@PDA. (a) TEM and SEM images of TPB-DMTP-COF, COF-Por, GA@PCOF, and GA@PCOF@PDA. Scale bar, 500 nm. (b) DLS size profiles and zeta potential of TPB-DMTP-COF, COF-Por, GA@PCOF, and GA@PCOF@PDA NPs in PBS (pH 7.4) at 25°C. (c) PXRD patterns of solid powders. (d) FT-IR spectra of solid powders. (e) N₂ adsorption and desorption isotherms at 77 K of TPB-DMTP-COF, COF-Por, GA@PCOF, and GA@PCOF@PDA. (f) UV-vis absorption spectra. Dispersant: water for TPB-DMTP-COF, PDA, and GA@PCOF@PDA; 1,4-dioxane for Por; and *N,N*-dimethylacetamide for GA. (g) Controlled GA leaching from GA@PCOF@PDA under different pH conditions.

crystal structure of pristine TPB-DMTP-COF was well maintained during the successive modifications. The formation of GA@PCOF@PDA was also supported by FT-IR analysis. Characteristic peaks at 1614 cm⁻¹ (C=N, for TPB-DMTP-COF), 798 cm⁻¹, and 3317 cm⁻¹ (the single-substituted Ar-H in-plane vibration and the pyrrole N-H, for Por), 1735 cm⁻¹ (for GA), and a wide band 3000–3500 cm⁻¹ (for PDA) were observed (Fig. 1d).

The porosity of GA@PCOF@PDA was studied by the nitrogen absorption and desorption isotherms at 77 K. According to the Brunauer-Emmett-Teller (BET) equation, the BET specific surface area (*S*_{BET}) of GA@PCOF@PDA was determined to be 520 m² g⁻¹, which is comparable to that of GA@PCOF (ca. 582 m² g⁻¹), indicating that the PDA modification occurred on the surface of GA@PCOF rather than in its cavities. However, their *S*_{BET} values are much lower than those of COF-Por (972 m² g⁻¹) and TPB-DMTP-COF (1039 m² g⁻¹), further demonstrating that the GA was in the COF pores (Fig. 1e).

GA@PCOF@PDA exhibited a broad absorption band in the range of 500–800 nm (Fig. 1f, Fig. S2). Besides the B- and Q-

bands of Por, a characteristic peak for GA at 362 nm was also observed but with a slightly decreased intensity when compared with GA@PCOF (Fig. S2). The Q-band absorption in the red range and the wide NIR absorption of GA@PCOF@PDA strongly supported the idea that it was a potential PDT and PTT nanoagent. In addition, the GA loading amount in GA@PCOF was calculated to be ca. 5.30 wt% based on the standard curve method (Fig. S3). Of note, the pristine TPB-DMTP framework has good stability toward different pH conditions, and no morphological change was observed in PBS with different pH values (pH 7.4 and 5.0) (Fig. 1a and Fig. S4), implying its prominent colloidal stability under physiological conditions [25]. In contrast, the GA release from GA@PCOF@PDA is pH-dependent, and 59.43% and 26.05% of GA guest were released under acidic (pH 5.0) and neutral conditions (pH 7.4), respectively, implying that this is a PDA coating-derived pH-response guest release process (Fig. 1g) [31].

Photodynamic and photothermal performance

The photodynamic behavior of GA@PCOF@PDA was evaluated

using DPBF as a $^1\text{O}_2$ probe. The absorbance of DPBF sharply declined to 40% after it was exposed to 660 nm irradiation (50 mW cm^{-2}) for 50 s, suggesting that the generation of $^1\text{O}_2$ was highly efficient (Fig. 2a). A similar absorbance change was obtained for both COF-Por and GA@PCOF, implying that the GA guest and PDA coating did not influence the $^1\text{O}_2$ generation of GA@PCOF@PDA. In contrast, essentially no decrease in DPBF absorbance was detected (2.8%, Fig. S5) with the 808-nm laser (2 W cm^{-2}) irradiation, indicating that this light source could not induce effective $^1\text{O}_2$ generation.

However, NIR light at 808 nm could effectively induce the photothermal conversion of GA@PCOF@PDA under physiological conditions. The photothermal conversion results of GA@PCOF@PDA under different conditions (concentration, 0–800 $\mu\text{g mL}^{-1}$; power density, 0.5–2.5 W cm^{-2}) are given in Fig. 2b, c, and the temperature increase was seen to be concentration- and laser density-dependent. For instance, when GA@PCOF@PDA ($200 \mu\text{g mL}^{-1}$) was irradiated with 808-nm laser (2 W cm^{-2}) for 10 min, the system temperature eventually surpassed 43°C , while only a 3.1°C temperature increase in the pure water was detected. Additionally, a negligible temperature change was observed after five laser irradiation cycles, demonstrating the good photothermal stability of GA@PCOF@PDA (Fig. 2d). The photothermal conversion efficiency of GA@PCOF@PDA was approximately 38.92% (Fig. 2e, Fig. S6) [32]. Notably, no light-induced effective temperature increase was observed for TPB-DMTP-COF, COF-Por, or GA@PCOF under the same conditions (Fig. 2f), suggesting that the PDA coating is responsible for the photothermal conversion in GA@P-

COF@PDA.

Intracellular $^1\text{O}_2$ generation

Intracellular $^1\text{O}_2$ generation was assessed using SOSG, which is a typical $^1\text{O}_2$ fluorescent probe. When compared with the intact and laser-irradiated 4T1 cells, a strong green fluorescence was observed in the groups treated with COF-Por and GA@PCOF after exposure to 660-nm laser (50 mW cm^{-2}) for 1 min, which was attributed to the COF-Por-mediated $^1\text{O}_2$ generation effect (Fig. 3a). Similar results were also detected in the GA@PCOF@PDA-treated group under the same photoradiation conditions. By contrast, the cells incubated with GA@PCOF@PDA after 808-nm laser irradiation (2 W cm^{-2}) for 1 min showed negligible effects on their intracellular $^1\text{O}_2$ levels. Taken together, these results validated the idea that GA@PCOF@PDA is capable of generating $^1\text{O}_2$ upon 660-nm laser irradiation.

GA@PCOF@PDA induced heat stress resistance reduction

To evaluate the ability of GA@PCOF@PDA to suppress the thermoresistance of cancer cells, 4T1 cells were incubated with free GA or GA@PCOF@PDA at 37 or 43°C for various periods of time, up to 4 h. After further incubation at 37°C for another 24 h, the relative cell viabilities were assessed using the standard MTT assay (Fig. 3b). For the untreated cells, viability was over 90% with the mild hyperthermia treatment at 43°C for 4 h. The relative viabilities for the free GA- or GA@PCOF@PDA-treated cells, however, were remarkably decreased under the same conditions. In contrast, neither free GA nor GA@PCOF@PDA exhibited significant cytotoxicity at 37°C , suggesting that they

Figure 2 Photodynamic and photothermal properties of the GA@PCOF@PDA. (a) $^1\text{O}_2$ generation of the different samples with different treatments (laser: 660 nm, 50 mW cm^{-2}): DPBF only (i), DPBF + laser (ii), DPBF + GA@PCOF@PDA (iii), DPBF + TPB-DMTP-COF ($160 \mu\text{g mL}^{-1}$, $200 \mu\text{g mL}^{-1}$ GA@PCOF@PDA equiv) + laser (iv), DPBF + COF-Por ($170 \mu\text{g mL}^{-1}$, $200 \mu\text{g mL}^{-1}$ GA@PCOF@PDA equiv) + laser (v), DPBF + GA@PCOF ($180 \mu\text{g mL}^{-1}$, $200 \mu\text{g mL}^{-1}$ GA@PCOF@PDA equiv) + laser (vi) and DPBF + GA@PCOF@PDA ($200 \mu\text{g mL}^{-1}$) + laser (vii). (b) Temperature increase caused by GA@PCOF@PDA with different concentrations upon 808-nm laser (2 W cm^{-2}) irradiation. (c) Temperature increase caused by GA@PCOF@PDA ($200 \mu\text{g mL}^{-1}$) under 808-nm laser with different power intensities. (d) The temperature variations of GA@PCOF@PDA ($200 \mu\text{g mL}^{-1}$) in PBS upon 808-nm laser (2 W cm^{-2}) irradiation for five cycles. (e) The temperature increase of GA@PCOF@PDA ($200 \mu\text{g mL}^{-1}$) in PBS upon 808-nm laser (2 W cm^{-2}) irradiation. (f) Comparison of the temperature increase induced by TPB-DMTP-COF ($160 \mu\text{g mL}^{-1}$, $200 \mu\text{g mL}^{-1}$ GA@PCOF@PDA equiv), COF-Por ($170 \mu\text{g mL}^{-1}$, $200 \mu\text{g mL}^{-1}$ GA@PCOF@PDA equiv), GA@PCOF ($180 \mu\text{g mL}^{-1}$, $200 \mu\text{g mL}^{-1}$ GA@PCOF@PDA equiv), and GA@PCOF@PDA ($200 \mu\text{g mL}^{-1}$) upon 808-nm laser (2 W cm^{-2}) irradiation.

Figure 3 Cooperative CT and phototherapy behavior of GA@PCOF@PDA at the cellular level. (a) $^1\text{O}_2$ generation in 4T1 cells under different treatments. 660 nm, 50 mW cm⁻², 1 min; 808 nm, 2 W cm⁻², 1 min. Scale bar, 100 μm. (b) Viabilities of the 4T1 cells cultured with GA@PCOF@PDA (100 μg mL⁻¹) and free GA (5 μg mL⁻¹) at 37 or 43°C for the specified time periods and then cultured at 37°C for a total incubation time of 24 h. All statistical analyses were performed using the Graphpad Prism 9.0 software, $n = 3$ samples per group. Significance was indicated as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, or **** $p < 0.0001$. (c) Western blot images for cell lysates of 4T1 cells under different treatments. (d) *In vitro* cytotoxicity of PCOF@PDA (i), GA@PCOF@PDA (ii), GA@PCOF@PDA + 808-nm laser (iii), GA@PCOF@PDA + 660-nm laser (iv), PCOF@PDA + 660- and 808-nm laser (v) GA@PCOF@PDA + 660- and 808-nm laser (vi) against 4T1 cells for 12 h. (e) Confocal images of calcein AM (green, live cells, $\lambda_{\text{ex}} = 488$ nm) and PI (red, dead cells, $\lambda_{\text{ex}} = 543$ nm) co-stained 4T1 cancer cells after various treatments. 660 nm, 50 mW cm⁻², 5 min; PTT: 808 nm, 2 W cm⁻², 10 min. Scale bar, 100 μm.

could really reduce the resistance of cells to heat stress.

Mechanism of the GA@PCOF@PDA induced heat resistance suppression

To further understand the mechanism for the reduced thermal resistance of cancer cells that was caused by GA@PCOF@PDA, western blot analysis was employed to examine the HSP90 expression in 4T1 cells with various treatments (Fig. S7). Compared with the 4T1 cells without any treatment (i, 56%), the 4T1 cells after 43°C incubation for 2 h displayed obviously

higher HSP90 expression levels (ii, 99%), which was consistent with previous reports [7], implying that the hyperthermia increased the levels of HSP90 expression. Significantly decreased HSP90 expression was observed in the GA-treated group at 43°C for 2 h (iii, 32%), validating the idea that GA can reduce heat resistance through the down-regulation of HSP90 expression. As expected, the expression of HSP90 remarkably increased in the PCOF@PDA-treated 4T1 cells at 43°C for 2 h (iv, 102%), whereas it sharply decreased (v, 19%) in the GA@PCOF@PDA-treated cells under the same conditions. These results suggested

the GA in GA@PCOF@PDA played a key role in suppressing HSP90 expression at elevated temperatures.

The expression of HSP90 in 4T1 cells under laser irradiation was then investigated (Fig. 3c). Compared with the control groups, including cells only (i, 81%), cells + PCOF@PDA (ii, 97%), cells + PCOF@PDA + 660-nm laser (iii, 95%), the HSP90 expression in the PCOF@PDA + 808-nm laser group significantly increased (iv, 138%). However, the cells treated with GA@PCOF@PDA + 808-nm laser (v, 29%) and GA@PCOF@PDA + 660- and 808-nm laser (vi, 23%) exhibited dramatically reduced levels of HSP90 expression. Therefore, we concluded that GA@PCOF@PDA could suppress the thermo-resistance of cancer cells *via* the inhibition of HSP90 expression, consequently allowing the PTT to kill cancer cells at the relatively low temperature of 43°C.

Cytotoxicity study

The cell killing efficacy of GA@PCOF@PDA was evaluated on 4T1 cells. As shown in Fig. 3d, for the cells treated with PCOF@PDA without GA loading (i), over 90% of the cells remained alive after incubation at 37°C for 12 h, even with PCOF@PDA concentrations of up to 400 $\mu\text{g mL}^{-1}$. The viability of the cells incubated with GA@PCOF@PDA (ii) visibly decreased with increased GA@PCOF@PDA concentrations under the same conditions, which is attributed to the CT of GA [33]. When 4T1 cells were incubated with GA@PCOF@PDA and then exposed to 808-nm NIR laser at a power density of 2 W cm^{-2} for 10 min (iii), (the cell culture temperature was approximately 43°C), the PTT monotherapy caused an evident change in cell viability, as it was 51.76%. Under 660-nm laser (50 mW cm^{-2} , 5 min) irradiation, PDT monotherapy (iv) gave an even lower 45.61% cell viability. In addition, the combination phototherapy (v) performed with PCOF@PDA under both 660- and 808-nm laser irradiation exhibited a reduced cell viability of 36.56%. By contrast, cells treated by GA@PCOF@PDA + both red and NIR lasers showed the most significantly reduced viabilities at 15.54%, indicating that the combination of low-temperature PTT, PDT, and CT could result in a superior effect for the inhibition of tumor cell proliferation. The plotting of the IC50 value for each group further supported the above conclusions (Fig. S8). As is shown, the treatment effect of combined PDT/PTT (vi, 200 $\mu\text{g mL}^{-1}$; 15.54%) is not the simple additivity of PTT monotherapy (iii, 200 $\mu\text{g mL}^{-1}$, 51.76%) and PDT monotherapy (iv, 200 $\mu\text{g mL}^{-1}$; 45.61%), but significantly stronger than any single therapy. The *in vitro* photocytotoxicity of GA@PCOF@PDA was also confirmed *via* a live/dead cell staining assay, in which tumor cells were co-stained with calcein-AM and PI to identify live and dead cells, respectively. The incubation of cells with GA@PCOF@PDA displayed the highest cytotoxicity as almost all fluorescence was observed to be red after NIR laser irradiation, when compared with other groups (Fig. 3e). These results are similar to those from the MTT assay, demonstrating the superior combination of low-temperature PTT, PDT, and CT in the constructed nanosystem towards cancer cells.

In vivo therapeutic effects of GA@PCOF@PDA on a primary tumor

Before evaluating the overall anticancer effects of the GA@PCOF@PDA, a hemolytic test was first conducted by incubating red blood cells with GA@PCOF@PDA at different concentra-

tions for 4 h. As shown in Fig. S9, no hemolysis was detected even for the concentration of GA@PCOF@PDA at 200 $\mu\text{g mL}^{-1}$; it might be attributed to the fact that GA release from GA@PCOF@PDA was pH-response guest release process and thus the concentration of GA was relatively low (Fig. 1g, Fig. S3). Such results demonstrated the good biocompatibility of GA@PCOF@PDA and it could be further applied for *in vivo* experiments. The *in vivo* antitumor ability was investigated in BALB/c mice with 4T1 tumors. When the tumor reached 100 mm^3 , the mice were randomly divided into seven groups ($n = 6$ per group), including PBS (i), PBS + 660- + 808-nm lasers (ii), GA@PCOF@PDA (iii), GA@PCOF@PDA + 808-nm laser (iv), GA@PCOF@PDA + 660-nm laser (v), PCOF@PDA + 660- + 808-nm lasers (vi), and GA@PCOF@PDA + 660- + 808-nm lasers (vii). The power densities of the 660- and 808-nm lasers were 50 mW cm^{-2} and 1.5 W cm^{-2} , respectively. The IR thermal camera was employed to record the *in vivo* photothermal heating profiles. The tumor site temperatures for the mice injected with GA@PCOF@PDA rapidly reached approximately 43°C under 808-nm laser irradiation, whereas the temperature of the mice injected with PBS only, exhibited a 6°C change (Fig. S10). The tumor volumes and total body weights of the mice were recorded every day throughout the treatment period. The tumors were collected and weighed on day 14. Significant tumor growth was observed in groups (i) and (ii) (Fig. 4a). Interestingly, we found that group (iii) showed tumor growth shrinkage. This is likely due to the chemotherapeutic action of GA, and is consistent with the observations in the MTT cell viability assay. For groups (iv) and (v), the tumor growth rate was partially suppressed due to the monotherapy of PTT and PDT, while obvious tumor suppression was observed in group (vi). Such results demonstrated the superior effect of the combination phototherapy over the monotherapy. As expected, 2 out of the 5 tumors in group (vii) completely disappeared and the other 3 tumors were of a very small size, confirming the enhanced anticancer efficiency of combination phototherapy at low temperatures. The photograph (Fig. S11) and weights of the tumors (Fig. 4b) collected from the different groups at the end of the treatment further confirmed these results. Notably, there were negligible body weight differences in the mice from the various groups (Fig. 4c), implying that there was no significant toxicity of GA@PCOF@PDA. The histopathological analysis of the main organ tissues, such as heart, liver, spleen, lung, and kidney (Fig. S12), from mice sacrificed after the treatment, also showed a high histocompatibility of GA@PCOF@PDA, as there was no severe cell damage in any of the groups. Additionally, H&E staining was carried out to investigate the phototherapy effect of GA@PCOF@PDA. H&E (Fig. 4d) and images of tumor sections showed that group (vii) could induce the most severe cell damage and led to a maximum ratio of cell apoptosis.

The HSP90 expression levels were then investigated *in vivo* using western blot analysis. When compared with the control groups, including PBS and PBS + 660- + 808-nm lasers, the relative HSP90 protein expression levels of the PCOF@PDA-treated group were obviously higher after the 808-nm laser irradiation, indicating that the PTT-induced heating stress *in vivo* could lead to the up-regulated expression of HSP90 (Fig. 4e, f). In contrast, the relative HSP90 protein expression levels in the GA@PCOF@PDA-treated group were much lower under the same administration, even with exposure to the photoradiation, suggesting that the heating stress was significantly relieved

Figure 4 Antitumor effects *in vivo*. (a) Time-dependent tumor growth curves for the mice with the different treatments: (i) PBS, (ii) PBS + 660- + 808-nm lasers, (iii) GA@PCOF@PDA, (iv) GA@PCOF@PDA + 808-nm laser, (v) GA@PCOF@PDA + 660-nm laser, (vi) PCOF@PDA + 660- + 808-nm lasers, and (vii) GA@PCOF@PDA + 660- + 808-nm lasers. (b) The corresponding weights of the tumors collected from the mice after finishing the administrated treatments. (c) Time-dependent body weight growth curves of the mice with the different treatments. All statistical analyses were performed using the Graphpad Prism 9.0 software. Significance was indicated as follows: * $p < 0.05$, ** $p < 0.01$ or *** $p < 0.001$, **** $p < 0.0001$. (d) H&E histological staining of the corresponding tumor tissues collected from the mice after finishing their treatments. Scale bar, 100 μ m. (e) The HSP90 expression in tumors collected from mice 24 h after various treatments as determined by western blot analysis. (f) Corresponding quantitative analysis of relative HSP90 protein expression levels with GAPDH as an internal reference in (e).

through the GA-mediated inhibition of HSP90.

***In vivo* therapeutic effects of GA@PCOF@PDA on lung metastasis**

It has been reported that hyperthermia in tumor tissues increases the risk of cancer metastasis [4,5]. The GA@PCOF@PDA-mediated low-temperature PTT and PDT may be favorable for treating lung metastasis breast cancer. To confirm the *in vivo* anti-metastasis efficacy of low-temperature phototherapy with GA@PCOF@PDA, the xenograft breast cancer model was established by planting 4T1 cells into the mammary fat pads of BALB/c mice (6–8 weeks old), which were then treated as described in Fig. 5a. Representative photographs for the lung tissues in each group of mice have been shown in Fig. 5b. The metastasis inhibition was evaluated by analyzing the histopathological changes in the lung H&E images (Fig. 5c) and recoding the number of lung metastatic nodules (Fig. 5d). As expected, tumor metastasis appeared to occur in the PBS group, whereas the mice treated with GA@PCOF@PDA plus PTT and

PDT exhibited the lowest levels of metastasis with fewer and smaller amounts of lung metastatic nodules. In addition, GA@PCOF@PDA plus PTT and PDT treatment also resulted in the smallest metastases area on the surface of the lung, as determined by quantitative statistical methods (Fig. 5e). Overall, these results have shown that GA@PCOF@PDA is capable of preventing breast cancer metastasis *in vivo*.

CONCLUSIONS

In summary, we have proposed a strategy to synthesize GA@PCOF@PDA nanocomposites using stepwise BDF, guest encapsulation, and surface modification processes for use in low-temperature cancer therapies. The obtained GA@PCOF@PDA can reverse the thermal resistance of tumor cells by inhibiting the expression of HSP90. Our approach constitutes, to our knowledge, the first example whereby enhanced phototherapy has been used to inhibit both primary and metastatic tumors at low temperatures. This method can be further applied

Figure 5 *In vivo* therapeutic effect of GA@PCOF@PDA on the suppression of cancer metastasis. (a) Schematic illustration of therapeutic treatments *in vivo*. (b) Representative lung images of 4T1 tumor-bearing mice after the following treatments: (i) PBS, (ii) (660 + 808) nm Laser, (iii) GA@PCOF@PDA, (iv) GA@PCOF@PDA + 808 nm Laser, (v) GA@PCOF@PDA + 660 nm Laser, (vi) PCOF@PDA + (660 + 808) nm Laser, (vii) GA@PCOF@PDA + (660 + 808) nm Laser. Green circles indicate lung metastases. (c) Representative H&E staining images (magnification $\times 20$) of the lung sections with the previously described treatments. (d) Quantitative analysis of lung metastases in (b). (e) Percentage of metastases in the lung in (c). All statistical analyses were performed using the Graphpad Prism 9.0 software. Significance was indicated as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ or **** $p < 0.0001$.

to other low-temperature-involved therapies, and also broaden the development of safe and effective strategies for the use of NCOF-based multifunctional nanomedicines in clinical applications.

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

Supplementary information Supporting data are available in the online version of the paper.

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纳米共价有机框架材料用于低温抗肿瘤生长和肺转移

冯杰^{†*}, 任文秀[†], 孔斐, 张策, 董育斌^{*}

摘要 光热疗法(PTT)在肿瘤治疗中有着广阔的应用前景。然而, 由于激光诱导的非特异性加热与热扩散现象的存在, 高温热(>50°C)在破坏肿瘤的同时可能导致肿瘤附近正常器官被灼伤, 给患者带来癌症复发和转移的风险。因此, 在低温加热下(≤45°C)有效破坏肿瘤对光学癌症治疗方法的未来临床转化具有重要价值。此外, 由于肿瘤的异质性和复杂性, 使得单模态PTT的治疗效果不佳, 需要发展联合抗肿瘤治疗策略。基于此, 我们采用逐步合成的方法, 依次通过键合缺陷功能化、客体封装和表面修饰步骤, 开发了一种高效的多模式纳米治疗剂GA@P-COF@PDA。该纳米制剂可在低温条件下实现光热/化疗/光动力联合治疗, 进而有效地抑制肿瘤的生长和转移。该研究表明, 我们可以通过共价有机框架(COF)纳米平台的多功能集成化, 实现在单一纳米平台上的多模式肿瘤治疗, 进而提高治疗效果。