Double-activation of mitochondrial permeability transition pore opening via calcium overload and reactive oxygen species for cancer therapy

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Abstract

Background: Calcium ions (Ca2+) participates in various intracellular signal cascades and especially plays a key role in pathways relevant to cancer cells. Mitochondrial metabolism stimulated by calcium overload can trigger the opening of the mitochondrial permeability transition pore (MPTP), which leads to cancer cell death.

Methods: Herein, a mitochondrial pathway for tumour growth inhibition was built via the double-activation of MPTP channel. Fe2+ doped covalent organic frameworks (COF) was synthesised and applied as template to grow CaCO3 shell. Then O2 was storged into Fe2+ doped COF, forming O2-FeCOF@CaCO3 nanocomposite. After modification with folic acid (FA), O2-FeCOF@CaCO3@FA (OFCCF) can target breast cancer cells and realize PDT/Ca2+ overload synergistic treatment.

Results: COF can induce the production of 1O2 under 650 nm irradiation for photodynamic therapy (PDT). Low pH and hypoxia in tumour microenvironment (TME) can activate the nanocomposite to release oxygen and Ca2+. The released O2 can alleviate hypoxia in TME, thus enhancing the efficiency of COF-mediated PDT. Abundant Ca2+ were released and accumulated in cancer cells, resulting in Ca2+ overload. Notably, the reactive oxygen species (ROS) and Ca2+ overload ensure the sustained opening of MPTP, which leads to the change of mitochondria transmembrane potential, the release of cytochrome c (Cyt c) and the activation of caspases 3 for cancer cell apoptosis.

Conclusion: This multifunctional nanosystem with TME responded abilities provided a novel strategy for innovative clinical cancer therapy.

Keywords: Ca2+ overload, MPTP, Hypoxia, Covalent organic frameworks, Photodynamic therapy
**Introduction**

Calcium signal plays an important role in various cancer progression processes such as proliferation, apoptosis and cell migration, which makes the regulation of calcium ions (Ca$^{2+}$) in cancer cells receive increasing attention [1]. As the second messenger, Ca$^{2+}$ has important effect on cell regulation, which can either induce cell survival or trigger apoptosis [2]. For instance, the fluctuations of Ca$^{2+}$ content in a certain range usually promote cell proliferation and survival, whereas sustained cytosolic calcium induced Ca$^{2+}$ overload can lead to cell apoptosis [3, 4]. Especially, tumor cells are more sensitive to Ca$^{2+}$ overload than normal cells [5–7]. Therefore, it is a promising strategy for triggering intracellular Ca$^{2+}$ overload to realise antitumor therapy.

As one of the intracellular Ca$^{2+}$ pools, mitochondrial uptakes Ca$^{2+}$ depending on the changes of transmembrane potential [8]. Mitochondrial permeability transition pore (MPTP), known as the mitochondrial megachannel, is a non-selective channel across the inner and outer layers of the mitochondrial membrane [9]. It has been reported that the continuous opening of MPTP is the direct cause of cancer cell apoptosis [10]. At physiological levels, Ca$^{2+}$ can activate the transient opening of MPTP, allowing protons or positive ions to enter the mitochondrial matrix to prevent excessive accumulation in the mitochondrial intermembrane space. However, MPTP can be activated to open continuously due to high [Ca$^{2+}$]m, high level of reactive oxygen species (ROS) and adenosine triphosphate (ATP) depletion, resulting in mitochondrial and cancer cells dysfunction [11–15].
Obviously, Ca\(^{2+}\) is the most important regulator and inductor for MPTP opening [16, 17]. CaCO\(_3\), a calcium-based biomineralized nanomaterial responding to the tumor acidic microenvironment with pH level as low as 6.2, has excellent degradability and good biocompatibility. As a natural Ca\(^{2+}\) reservoir, CaCO\(_3\) can provide sufficient Ca\(^{2+}\) source during the process of tumor treatment [18–23]. Moreover, when mitochondrial Ca\(^{2+}\) overload occurs, ROS can promote Ca\(^{2+}\) to stimulate sustained MPTP opening [24].

Photodynamic therapy (PDT) is a photosensitized reaction with biological effects, which involves oxygen molecules. The process is that the photosensitizer (PSs) absorbed by the tissue is excited by laser irradiation of a specific wavelength, and the excited PSs transfer energy to the surrounding oxygen to produce singlet oxygen (\(^{1}\text{O}_2\)) with strong activity. \(^{1}\text{O}_2\) reacts with adjacent biological macromolecules to produce cytotoxicity, resulting in cell damage and even death [25–27]. Compared with conventional treatments such as surgery, chemotherapy and radiotherapy, PDT has the advantages of less trauma, low toxicity and high selectivity [28–30]. However, PDT-mediated continuous oxygen consumption would aggravate hypoxia of cancer cells, which further blunts the therapeutic effect of PDT [31, 32]. Therefore, oxygen storage materials, including hemoglobin [33], perfluorocarbon [34] and metal organic framework (MOF) [35], are used to alleviate hypoxia in tumors.

As an emerging type of porous crystal material, covalent organic frameworks (COF) have been studied for cancer treatment in addition to their conventional applications in energy storage, catalysis, sensing and separation [36]. Owe to their unique structure and characteristics such as porosity, stability and biocompatibility, COF has been widely applied for drug delivery, photodynamic therapy and photothermal therapy [37–40], Tan and his colleagues, for example, reported a porphyrin-COF for photodynamic therapy of tumors. Under near-infrared irradiation, COF nanoparticles produced abundant ROS to induce cancer cell apoptosis result in their crystalline network structure [41]. Li and his colleagues synthesized a porous 8-hydroxyquinoline functionalized organic covalent framework (COF-HQ) with pH-sensitive tumor microenvironment for 5-FU loading, achieving efficient drug delivery and anti-tumor effects [42]. To the best of our knowledge, the use of COF as a smart carrier for oxygen molecules to achieve tumor-targeted transport has not been reported.

Herein, we synthesized Fe\(^{2+}\)-doped COF by Schiff base reaction at room temperature. Then FeCOF was used as a template for the growth of FeCOF@CaCO\(_3\) by gas diffusion method. And folic acid (FA) was modified on the surface of FeCOF@CaCO\(_3\) to form FeCOF@CaCO\(_3\)@FA (FCCF) for achieving targeted therapy against breast cancer. FCCF possessed highly efficient oxygen-carrying capacity owing to the affinity of doped Fe\(^{2+}\) with oxygen. Upon internalization into breast cancer cells, O\(_2\)-FeCOF@CaCO\(_3\)@FA (OFCCF) nanoparticles were disassembled, releasing O\(_2\) and Ca\(^{2+}\). COF, as the photosensitizer, was used to produce ROS under light exposure. The released O\(_2\) could enhance COF-mediated PDT in hypoxic tumour microenvironment (TME). Moreover, the coated CaCO\(_3\) layer exhibited excellent pH-dependent dissociation behavior, causing rapid release of Ca\(^{2+}\) under the acidic conditions. Large amounts of Ca\(^{2+}\) could accumulate in mitochondria, leading to the disruption of Ca\(^{2+}\) homeostasis and mitochondria dysfunction. Therefore, the high levels of ROS and intracellular accumulation of Ca\(^{2+}\) induced the continuous opening of MPTP, causing an influx of Ca\(^{2+}\) to kill cancer cells. Besides, pathological and biochemical tests confirmed that OFCCF nanoparticles could avoid the disturbance of systemic toxicity (Scheme 1).

Results

Synthesis and characterization of O\(_2\)-FeCOF@CaCO\(_3\)@FA NPs

The well-dispersed spherical FeCOF was simply synthesized by using acetic acid as catalyst at room temperature (Fig. 1a and Additional file 1: Figure S1). FeCOF@CaCO\(_3\) was prepared via gas diffusion method. As illustrated in Fig. 1b, in a closed vacuum, the CO\(_2\) and NH\(_3\) gases produced by the natural decomposition of NH\(_4\)HCO\(_3\) would continuously diffuse into the mixed solution containing Ca\(^{2+}\). Simultaneously CO\(_3^{2-}\) was provided into the alkaline solution to trigger the formation of CaCO\(_3\) [43]. Thin nanosheets of CaCO\(_3\) could be observed on the surface of FeCOF by transmission electron microscopy (TEM, Fig. 1c) and scanning electron microscopy (SEM, Additional file 1: Figure S2). Elemental mapping analysis showed that C, O, N, Fe and Ca elements of FCCF nanoparticles were homogeneously distributed (Fig. 1d). X-ray photoelectron spectroscopy (XPS) was used to analyse Ca element signal of FeCOF@CaCO\(_3\). The two peaks located at 347.2 eV and 350.7 eV for 2p\(_{1/2}\) and 2p\(_{3/2}\) respectively, were considered as the characteristic peaks of CaCO\(_3\) (Fig. S3) [44]. Powder X-ray diffraction (PXRD) patterns have confirmed the excellent crystallinity of FeCOF and FeCOF@CaCO\(_3\) (Additional file 1: Figure S4) [37, 45]. The surface area and pore volume of FeCOF and FeCOF@CaCO\(_3\) were 1380.170 m\(^2\)/g, 1.292 cc/g and 373.731 m\(^2\)/g, 0.305 cc/g. Compared with FeCOF, FeCOF@CaCO\(_3\) showed decreased Brunauer–Emmett–Teller (BET) surface area and pore volume, confirming the successful coating of CaCO\(_3\) (Additional file 1: Figure S5). Then FA was modified on the surface
of FeCOF@CaCO₃ to obtain the final FeCOF@CaCO₃@FA (FCCF) nanoparticles (Fig. 1e). In the FTIR spectra of FeCOF@CaCO₃, the peak at 1592 cm⁻¹ (peak 1) was ascribed to the vibration of -NH₂ and the characteristic peak at 712 cm⁻¹ (peak 2) belonged to carbonate. In the FTIR spectra of FCCF, the characteristic peaks at ~1573 (peak 3) and 1647 cm⁻¹ (peak 4) have been characterized as the stretching vibration mode of secondary amide bonds (C=O-NH), further demonstrating the successful synthesis of FCCF [46, 47]. UV-visible (UV-vis) absorption spectrum of FCCF showed that there was a broad absorption between 400 and 700 nm, which was similar to the absorption of FeCOF nanoparticles. Meanwhile, the strong absorbance peak centered at 281 nm of FCCF validated the integration of FA into the nanosystem (Fig. 1g). TEM and Dynamic light scattering (DLS) measurements showed that the mean size of FCCF was about 230 nm (Additional file 1: Figure S6 and Table S1), slightly larger than that of FeCOF and FeCOF@CaCO₃ nanoparticles. In addition, the successful synthesis of FCCF could also be proved by the zeta potential changes (Additional file 1: Figure S7). The post-modification of FA could enhance the bio-compatible and tumor targeting abilities of FCCF. As shown in Additional file 1: Figure S8, FCCF composite were uniformly dispersed in water, phosphate buffered saline (PBS) and cell culture medium (containing 10% serum) for 7 days without any aggregation at room temperature. The morphology of the nanocomposite had no change even after incubation in water for a week. These results verified the good stability of FCCF for potential clinical applications.
In vitro release study
In order to verify TME-activated bio-decomposition abilities of FCCF nanoparticles, various measurements were conducted. FCCF and FeCOF nanoparticles were dispersed in PBS with different pH to observe the morphology changes at different time points. As shown in Additional file 1: Figure S9 and S10, FCCF nanoparticles showed no change on size and structure when dispersed in PBS at pH 7.4. In contrast, FCCF nanoparticles dissociated in PBS with low pH (6.5 and 5.5), resulting in the nanosheet like morphology of the surface was largely lost after 1 h. Meanwhile, the hydrodynamic sizes of nanoparticles for time-dependent changes were monitored by DLS at different pH values. The hydrated particle of FCCF nanoparticles at pH 6.5 and 5.5 for 1 h were around 180 nm and 100 nm, respectively (Fig. 2a). Then, the time-dependent release profiles of Ca\(^{2+}\) were further assessed in buffers with various pH values. As shown in Fig. 2b, the low pH condition (5.5) led to a sustained release of Ca\(^{2+}\), with 89.2% Ca\(^{2+}\) being released from FeCOF carrier. However, only about 66.4% Ca\(^{2+}\) was released after being incubated at pH 6.5. The above results strongly proved that FCCF exhibited promising pH-responsive Ca\(^{2+}\) release ability.

The release of Ca\(^{2+}\) further facilitated oxygen diffusion to meet the increased oxygen demand in hypoxic tumor. O\(_2\) releasing ability of OFCCF and O\(_2\)-COF in deoxidized PBS buffer was illustrated in Fig. 2c, d. The stored O\(_2\) of OFCCF and O\(_2\)-COF were released under a hypoxic environment by passive transportation. However, the released O\(_2\) from OFCCF was approximately twofold as that from O\(_2\)-COF, which was attributed to the affinity of Fe\(^{2+}\) with O\(_2\). Moreover, the bubbles produce in the OFCCF solution also demonstrated the specific oxygen release behavior of Additional file 1: Figure S11. The ROS generation ability of OFCCF was investigated by
1,3-diphenylisobenzofuran (DPBF). Compared with free DPBF, COF showed effective time-dependent ROS production under 650 nm (0.72 W cm\(^{-2}\)) laser irradiation, which was attributed to its strong absorption (Fig. 1g). Moreover, the released O\(_2\) could enhance the production of ROS (Fig. 2e). Next, electron spin resonance (ESR) with 2,2,6,6-tetramethyl-4-piperidinol (TEMP) as singlet \(\text{^1}O_2\) trapping agent was used for detecting \(\text{^1}O_2\) generation. \(\text{^1}O_2\) signal (1:1:1) was observed for OFCCF under 650 nm laser irradiation, which was stronger than that of FCCF (Fig. 2f). Overall, OFCCF with excellent \(\text{^1}O_2\) generation ability could effectively overcome tumor hypoxia and enhance PDT effect for breast cancer.

**Cancer cell death induced by calcium overload**

Next, the cellular internalization of rhodamine B (RhB)-labelled FCCF nanoparticles was examined in murine 4T1 breast cancer cells. Figure 3a indicated the time-dependent internalization process of FCCF nanoparticles, as evidenced by the colocalization of red fluorescence for RhB-labelled FCCF nanoparticles and the green fluorescence for LysoTracker. These results improved that FCCF nanoparticles could be effectively uptaken by 4T1 cells, which was beneficial to killing tumor cells. Within acidic lysosomes, OFCCF nanoparticles could release Ca\(^{2+}\) rapidly, which led to a direct increase of osmotic pressure and influx of Cl\(^{-}\) and H\(_2\)O molecules to result in proton sponge effect \([21, 46, 47]\). Simultaneously, under 650 nm laser irradiation, a large number of \(\text{^1}O_2\) were produced by the nanoparticles, causing the destruction of lysosome membrane structure to favor the endosomal escape of nanoparticles. Then, intracellular Ca\(^{2+}\) concentration was monitored using the calcium indicator dye Fluo-4. Weak green fluorescence was observed in PBS group and L group of 4T1 cells. In contrast, compared with other groups, the intracellular Ca\(^{2+}\) concentration in the OFCCF group was highest (Additional file 1: Figure S12). In addition, mitochondrial Ca\(^{2+}\) concentrations were quantified using the calcium indicator dye Rhod-2. The FCCF, COF+ and CaCO\(_3\) groups exhibited weak red luminescence, while the group treated with OFCCF+ exhibited strong intracellular luminescence, indicating Ca\(^{2+}\) influx of 4T1 cells could be better activated in the presence of ROS (Additional file 1: Figure S13). Via Calcium Colorimetric assay, the OFCCF+ displayed the highest intracellular Ca\(^{2+}\) concentration as 19.4 \(\mu\)g/mL, indicating an obvious Ca\(^{2+}\) overloading (>3.2 \(\mu\)g/mL in PBS group) (Additional file 1: Figure S14). The release of Ca\(^{2+}\) and the hypoxic tumor microenvironment would trigger the free diffusion of oxygen by
Fig. 3  
(a) The images of 4T1 cells incubated with FCCF recorded at different time points. b [Ru(dpp)₃]Cl₂ was used as probe to detect O₂ generation after treatment with: (1) PBS+, (2) FCCF, (3) FCCF+ and (4) OFCCF+. c Western blot analysis of HIF-1α expression of 4T1 cells. d Mitochondrial distribution and mitochondrial membrane potential images of 4T1 cells after different treatments. e Bio-TEM images of 4T1 cells after different treatments. Red arrows exhibit the location of destructed mitochondria in 4T1 cells.
passive-transport. The O$_2$ probe [Ru(dpp)$_3$]Cl$_2$ (RDPP) which is prone to luminescence quenching by oxygen was used to monitor cellular O$_2$-evolving. As shown in Fig. 3b, the green fluorescence intensity of OFCCF group decreased obviously under hypoxic conditions, while the green fluorescence was observed for L treated and FCCF treated groups. Then, quantitative analysis of dynamic changes of intracellular oxygen via calculating average intensity by ImageJ software. The green fluorescence intensity of 4T1 cells treated with OFCCF+ was 22%, which was about three times lower than the other three groups (Additional file 1: Figure S15). Meanwhile, as the expression of HIF-1α protein is upregulated under a hypoxic condition, the degree of hypoxia can be further assessed according to the level of HIF-1α. The OFCCF treated 4T1 cells exhibited a low expression of HIF-1α by western blotting (WB) analysis (Fig. 3c and Additional file 1: Figure S16). These results suggested that OFCCF could release O$_2$ to alleviate the hypoxic state of tumor microenvironment. The enhance of ROS generation by OFCCF-mediated O$_2$ was proved by intracellular 1O$_2$ test with 2,7’-dichlorodihydrofluorescein diacetate (DCFH-DA) fluorescence as a probe. As shown in Additional file 1: Figure S17, a comparative intensity of green fluorescence was observed under hypoxia and normoxia conditions, suggesting the key role of oxygen release in PDT.

Ca$^{2+}$ overload and 1O$_2$ can cause the conformational changes of MPTP structural proteins, which allows substances with a molecular weight greater than 1500 to pass through the inner mitochondrial membrane (IMM) by non-selectively way. The entered substances result in the collapse of mitochondrial membrane potential (MMP), the uncoupling of oxidative phosphorylation process and the disturbance of ATP production, consequently causing mitochondria function impairment [16]. Therefore, we used commercial Calcein-AM or Calcein-AM + CoCl$_2$ dye as a fluorescence indicator to evaluate whether OFCCF was able to induce the continuous activation of MPTP. Upon treatment with FCCF or COF +, green fluorescence was observed. In contrast, no green fluorescence was detected for OFCCF group. However, upon receiving simultaneous exposure of OFCCF+ and 5 µM uncoupling agent (CCCP), the 4T1 cells exhibited green fluorescence signal (Additional file 1: Figure S18), suggesting the critical role of Ca$^{2+}$ overloading to activate MPTP opening. Next, to reveal the degree of mitochondrial damage induced by enhanced mitochondrial Ca$^{2+}$ overload, the mitochondrial membrane potential of 4T1 cells was evaluated using commercial JC-1 dye. The OFCCF+ group showed strong green fluorescence signal, in marked contrast to the strong red fluorescence observed in other groups. Subsequently, intracellular mitochondrial distribution was detected by staining with Mito Tracker® Red CMXRos. As expected, the fewest mitochondria damage were detected in the OFCCF + group (Fig. 3d and Additional file 1: Figure S19). Finally, biological transmission electron microscopy (Bio-TEM) was used to visualize changes in their mitochondria (Fig. 3e). The 4T1 cells treated with FCCF, COF + or FCCF+ exhibited only mild mitochondrial destruction, whereas OFCCF+ caused the most obvious mitochondrial destruction, with visible swelling and cavitation of mitochondria. These results confirm that OFCCF+ can cause severe mitochondrial damage through mitochondrial Ca$^{2+}$ overload. In addition, the intracellular ATP content treated with OFCCF+ decreased significantly in comparison to other five groups, which was partially produced via the oxidative phosphorylation inside the mitochondria (Additional file 1: Figure S20). All of the results revealed that the increased intracellular Ca$^{2+}$ and production of 1O$_2$ induced by OFCCF+ could cause mitochondrial dysfunction.

The collapse of MMP induces a series of pathological changes in mitochondria, resulting in the release of Cyt c from mitochondrial matrix into cytoplasm. And Cyt c and apoptotic protein activator-1 (Apaf-1) can form a composite to trigger cell apoptosis [48]. To confirm this principle, the expression of apoptosis-related proteins was investigated by western blotting. As illustrated in the Fig. 4a, the expression levels of Cyt c and caspase 3 increased after the treatment of OFCCF+ in comparison to the PBS, FCCF and FCCF+ groups. By contrast, the protein levels of Bcl-2 in 4T1 cells were markedly down-regulated after OFCCF+ treatment. Detailed quantitative results of WB were gathered in Fig. 4b. In addition, the release of Apaf-1 from the supernatant of 4T1 cells was measured by enzyme-linked immunosorbent assay (ELISA). As shown in Fig. 4c, the OFCCF+ treatment elevated the release of Apaf-1 effectively. The above results indicated that mitochondrial-mediated apoptotic pathway was activated after the mitochondrial Ca$^{2+}$ overload and production of 1O$_2$.

Then, the cytotoxic effect of COF and FCCF nanoparticles towards L929 fibroblast cells and 4T1 cells was evaluated using the standard methyl thiazolyl tetrazolium assay (MTT). L929 cells were treated with various concentrations of COF and FCCF nanoparticles within 24 and 48 h, the survival rate maintained above 80%. The proliferation ability of L929 cells treated with OFCC+ and OFCCF+ decreased slightly (Additional file 1: Figure S21). These results demonstrated the biosafety and biocompatibility of the nanoparticles. To further investigate the therapeutic effect of Ca$^{2+}$ overload/PDT, the anti-cancer effect of OFCCF under hypoxic and normoxic environment was tested. Upon 650 nm laser irradiation, the cytotoxicity of FCCF+ under
normoxic environment was greatly enhanced than that of FCCF+ under the hypoxic environment. Notably, the hypoxia condition had significant effect on the cancer cell killing effect of FCCF+-treated group. While the survival rate of OFCCF+ treated group was almost the same as the group under hypoxia condition owing to the oxygen-carrying properties of FeCOF (Fig. 4d). Moreover, from the live/dead cell staining test, we observed that most cells remained alive after the treatment of PBS and L. By contrast, the OFCCF+ treated group showed a large number of dead cells in the Additional file 1: Figure S22. This result was consistent with the flow experimental results (Additional file 1: Figure S23). Collectively, all above results reveal that OFCCF is a promising candidate to induce cancer cell death through Ca2+ overload and 1O2 co-activating MPTP opening (Fig. 4e).

**In vivo therapeutic effect investigation**

The in vivo therapeutic efficacy of OFCCF was further examined by a 4T1 breast tumor model. The tumor-bearing mice were randomly divided into six groups for the different treatments: (1) PBS, (2) 650 nm laser irradiation (L), (3) FCCF, (4) COF+, (5) FCCF+ and (6) OFCCF+. After intravenously injection with nanocomposites (100 μg mL−1) for 12 h, the mice were treated with 650 nm laser irradiation (0.72 W cm−2) for 5 min (Fig. 5a). The obtained nanocomposites had no side effect on the body weight of mice, suggesting the good biosafety of the nanocomposites (Additional file 1: Figure S24). Tumor suppression assessments showed a considerable suppression effect on the tumors of FCCF+ group and OFCCF+ group in comparison with other four groups (Fig. 5b). This result was consistent with the pictures of mice tumors and tumor weight (Fig. 5c and Additional file 1: Figure S25). Furthermore, hematoxylin and eosin (H&E) staining results were shown in Fig. 5d, obvious cell necrosis and apoptosis could be observed in OFCCF+ group. From the TUNEL staining results, the green signal represented that cells apoptosis was appeared after the treatment of OFCCF+. Meanwhile, the typical morphology of apoptotic cells was detected in caspase 3 staining assay. Finally, to better assess the effect of this synergistic therapy, we next examined the lung metastasis of 4T1 tumor-bearing mice after various
treatments. H&E staining and imaging of lung sections confirmed that OFCCF + effectively suppressed tumor metastasis (Additional file 1: Figure S26).

Moreover, the therapeutic efficiency of OFCCF + was further evaluated (Fig. 6a). To study the bio-distribution of FCCF/FCCF was injected intravenously into 4T1-tumor-bearing mice and Fe content was tracked by inductively coupled plasma mass spectrometry (ICP-MS). As shown in Fig. 6b, nanoparticles preferentially accumulated at the tumor site due to the specific binding of FA with breast cancer folate receptors, showing the highest content at 12 h after injection. Apart from tumors, the nanoparticles were also accumulated in liver and kidney. As shown in Fig. 6c, the content of FCCF nanoparticles in liver and kidney decreased gradually, confirming that they could be metabolized from the body through the renal-urinary clearance system within one week. The tumor accumulation and clearance performance of FCCF provides enormous potential for effective and safe cancer treatment. Furthermore, the
bio-degradation performance of nanoparticles triggered by TME was further studied. After the PBS, FCCF and OFCCF were injected intravenously into 4T1-tumor-bearing mice for 12 h, respectively, the Ca\(^{2+}\) released from nanoparticles accumulated in tumor owing to the acidic condition of TME. As we expected, the Ca\(^{2+}\) content in the tumors significantly increased after being treated with OFCCF + in comparison to FCCF and FCCF + groups. It is further revealed that the production of ROS could promote the aggregation of Ca\(^{2+}\), resulting in the apoptosis caused by Ca\(^{2+}\) overload (Fig. 6d). Next, the possible mechanism of Ca\(^{2+}\) overload-induced apoptosis was explored by ELISA and western blotting to determine the content of apoptosis-related proteins of tumours. Once Cyt c is released from the mitochondria, it can couple with Apaf-1 to form the apoptosome. As illustrated in Fig. 6e, the upregulation of Apaf-1 confirmed the formation of apoptosome. Besides, the expression levels of Cyt
c, Bcl-2 and caspase 3 were further detected by western blotting. As illustrated in the Fig. 6f, compared with the control groups (PBS, FCCF and FCCF+), Cyt c and caspase 3 expressions levels increased significantly but the protein levels of Bcl-2 decreased after OFCCF+ treatment. The quantitative analysis of western blotting was shown in Additional file 1: Figure S27. Compared with the other groups, the ATP level of OFCCF+ group decreased significantly owing to Ca2+ overload in mitochondria, which would damage the energy supply of cancer cells (Additional file 1: Figure S28). To identify the ability of OFCCF to relieve tumor hypoxia, mice tumors were obtained for HIF-1α (green) staining assay after being injected with the nanoparticles for 10 h. As illustrated in the Fig. 6g, the intensity of fluorescence was hardly visible in OFCCF+ group. For ROS staining assay, OFCCF+ group showed the strongest DCFH-DA fluorescence signals in tumor slices, which was consistent with the HIF-1α (green) staining assay results. Meanwhile, the remission of tumor hypoxia was further verified by down-regulation of HIF-1α expression level, indicating that released oxygen could relieve intratumoral hypoxia and enhance production of ROS (Additional file 1: Figure S29).

To assess the potential therapeutic toxicity of FCCF, a systemic toxicity study was performed. The physiological pathology of main organs were analysed by H&E staining. No significant pathological changes in major organs (heart, liver, kidney lung, and spleen) were observed after different treatments (Additional file 1: Figure S30), suggesting the excellent biosafety of the nanocomposite in vivo. Meanwhile, biochemical analysis further confirmed that OFCCF+ had low toxicity (Additional file 1: Figure S31).

Conclusion

In summary, we have successfully prepared an OFCCF nanocomposite with two-way activation of MPTP opening by ROS and Ca2+ overload for precise targeted therapy of breast cancer. OFCCF with tumor-targeting ability could effectively aggregate at the tumor sites. The subsequent rapid release of Ca2+ and oxygen were attributed to the hypoxia and low pH of TME. Overflowed Ca2+ rapidly accumulated inside the cytoplasm, which led to Ca2+ influx and activated MPTP opening. Upon NIR light irradiation, the released oxygen could enhance the effect of COF-mediated PDT, and the generation of ROS could promote the activation of MPTP opening. Notably, these two activation mechanisms ensured the sustained opening of MPTP, leading to the change of mitochondria transmembrane potential, release of Cyt c and activation of caspases 3, thus inducing cell apoptosis. Additionally, for the first time, we utilized FeCOF with hypoxia responsive as oxygen carrier, alleviating tumor hypoxia and enhance PDT efficiency. More importantly, OFCCF nanocomposites based treatment has been proved to be a safe and effective strategy to inhibit tumor growth and open new directions for clinical cancer therapy.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12951-022-01392-y.
hepatic function indicators and Blood urea nitrogen (BUN) as renal function indicators were measured. Blood hematological counts: Blood levels of white blood cells (WBC), red blood cells (RBC), Hemoglobin (HGB), Hematocrit (HCT), Mean corpuscular volume (MCV), Mean corpuscular hemoglobin (MCH), Mean corpuscular hemoglobin concentration (MCHC) and Blood platelets (PLT). Data are presented as mean ± SD (n = 4).

Acknowledgements
Not applicable.

Author contributions
These authors contribute equally. Correspondence: MP, Y.Z and CZ. Conceptualization, MP, Y.Z and CZ; writing—original draft preparation, Y.Z and S.J.; experiments performing, all authors. All authors read and approved the final manuscript.

Funding
This project is financially supported by the National Natural Science Foundation of China (NSFC 21471145), Science and Technology Development Planning Project of Jilin Province (201701011729JC), and “Hundred Talents Program” of the Chinese Academy of Sciences.

Availability of data and materials
The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

Declarations

Ethics approval and consent to participate
All animal experiments were conducted strictly following the guidelines from the Animal Protection and Use Committee of Jilin University.

Consent for publication
All authors have approved the manuscript be submitted.

Competing interests
All authors have no competing interest in regard to this work.

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Received: 24 December 2021 Accepted: 21 March 2022 Published online: 12 April 2022

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