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Communication

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Nanoscale Metal-Organic Frameworks Stabilize Bacteriochlorins for Type I and Type II Photodynamic Therapy

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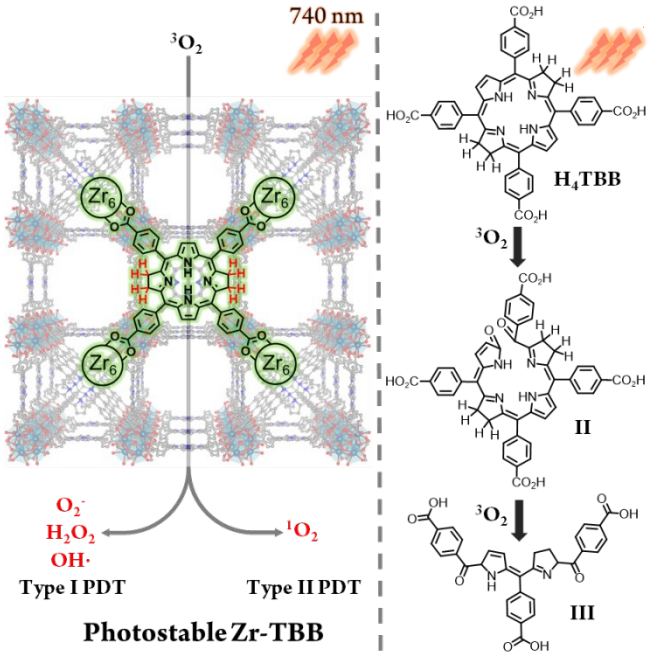
Supporting Information Placeholder

ABSTRACT: Herein we report the design of a bacteriochlorin-based nanoscale metal-organic framework, Zr-TBB, for highly effective photodynamic therapy via both type I and type II mechanisms. The framework of Zr-TBB stabilizes 5,10,15,20-tetra(*p*-benzoato)bacteriochlorin (TBB) ligands toward oxygen and light via geometrical constraint. Upon 740 nm light irradiation, Zr-TBB efficiently generates various reactive oxygen species, including singlet oxygen, superoxide anion, hydrogen peroxide and hydroxyl radicals to afford superb antitumor efficacy on mouse models of breast and colon cancers with cure rates of 40% and 60%, respectively.

Photodynamic therapy (PDT) is a minimally invasive and effective local therapy for many cancers,[1-5] but its clinical utility is limited by side effects from photosensitivity caused by residual photosensitizers (PSs) in normal tissues, shallow light penetration depth in tumors, and low oxygen concentrations in hypoxic tumors.[6-8] As highly reduced derivatives of porphyrins and chlorins, bacteriochlorins possess several distinct features to overcome the challenges faced by conventional PSs: 1) weak absorption in the visible spectrum minimizes photosensitivity from ambient light, 2) strong absorption in the near-infrared region (700-850 nm) increases PDT efficacy, and 3) type I PDT tolerates hypoxia.[9-11] Padeliporfin, a Pd-coordinated bacteriochlorin, was approved in Europe for PDT treatment of prostate cancer.[12]. However, bacteriochlorins are unstable toward oxygen and light,[13-15] significantly reducing their potency in PDT.[16-18]

With tunable and porous structures,[19-22] high PS loading,[23] and rigid structures,[24] nanoscale metal-organic frameworks (nMOFs) have emerged as novel nanophotosensitizers for PDT.[25-28] By incorporating different PS ligands, nMOFs can be fine-tuned to optimize PDT efficacy. The rigid frameworks of nMOFs not only constrain the ligands from structural changes to reduce unimolecular photodecomposition but also isolate the PSs from each other to prevent inter-PS self-quenching.

Herein we report the use of nMOFs to stabilize bacteriochlorins for effective PDT. Experimental and computational studies demonstrated the stabilization of 5,10,15,20-tetra(*p*-benzoato)bacteriochlorin (TBB) ligands in the Zr-TBB nMOF toward oxygen and light owing to geometrical constraint by the framework. Zr-TBB mediated effective PDT via both type I and type II mechanisms by generating various reactive oxygen species (ROSs), including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (·OH), and singlet oxygen ($^1O_2$), upon irradiation at 740 nm (Scheme 1). Zr-TBB showed superb *in vivo* antitumor efficacy on 4T1 and MC38 bearing mouse models of breast and colon cancers to afford cure rates of 40% and 60%, respectively.



Scheme 1. Stabilization of bacteriochlorin ligands in Zr-TBB for type I and type II PDT.

The new bacteriochlorin H_4TBB was synthesized via solvent-free reduction of 5,10,15,20-tetra(*p*-benzoato)porphyrin (H_4TBP) with *p*-toluenesulfonyl hydrazide (Figure S1).[29] The UV-vis spectrum of H_4TBB in N,N-dimethylformamide (DMF) exhibited four major peaks (Figure 1e) assignable to the transitions from two HOMOs (HOMO-1 and HOMO) to two LUMOs (LUMO and LUMO+1) based on four-orbital model.[30] For H_4TBB, the B_y peak at λ_{max} = 361 nm had a molar extinction coefficient (ε) of 70.4mM^{-1}·cm^{-1} whereas the B_x peak at λ_{max} = 377 nm had an ε of 73.4mM^{-1}·cm^{-1}. These ε values are ~4 times lower than those of Soret bands in H_4TBP (ε_{420} = 460 mM^{-1}·cm^{-1}) and 5,10,15,20-tetra(*p*-benzoato)chlorin (H_4TBC, ε_{420} = 381 mM^{-1}·cm^{-1}), suggesting that

H_4TBB might alleviate photosensitivity side effects from ambient light.[26] The Q_x and Q_y peaks of H_4TBB had an ε_{521} of 32.4 $mM^{-1}\cdot cm^{-1}$ and an ε_{742} of 58.4$mM^{-1}\cdot cm^{-1}$, respectively. The Q_y peak of H_4TBB at 742 nm is nearly ideal for tissue penetration, and H_4TBB has ~12 and ~2 times higher ε values than those of H_4TBP and H_4TBC, respectively. [26] H_4TBB is thus a superior PS over H_4TBP and H_4TBC with an optimal Q_y wavelength and a much higher ε.[31]

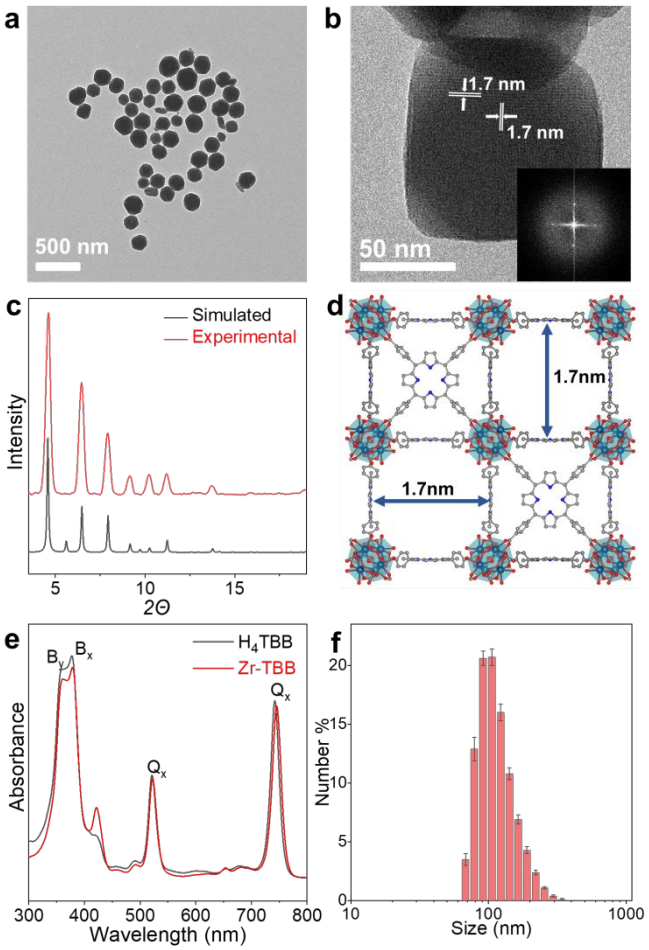


Figure 1. TEM image (a), HR-TEM image and FFT pattern (inset) (b), PXRD pattern (c), X-ray crystal structure (d), UV-vis spectra in DMF (e), and DLS number-averaged diameter in EtOH (f) of Zr-TBB.

Zr-TBB was synthesized via a solvothermal reaction of $ZrCl_4$, H_4TBB, and 88% formic acid in DMF at 100 ℃ under anaerobic conditions. Single crystal X-ray diffraction of Hf-TBB revealed a PCN-224 structure in the $Im\bar{3}m$ space group with $Hf_6(\mu_3\text{-}O)_4(\mu_3\text{-}OH)_4$ SBUs linked by TBB ligands to afford a 3-D framework of *she* topology (Figure 1d and Figure S3).[32] Powder X-ray diffraction pattern (PXRD) studies indicated that Zr-TBB adopted the same structure as Hf-TBB (Figure 1c) with a formula of $[Zr_6(\mu_3\text{-}O)_4(\mu_3\text{-}OH)_4(OH)_6(H_2O)_6]_2(TBB)_3$. Inductively coupled plasma-mass spectrometry (ICP-MS) and UV-vis spectra gave a Zr to TBB ratio of 4.22, which is slightly lower than the theoretical ratio of 4 likely due to minor decomposition of TBB ligands during nMOF synthesis. Thermogravimetric analysis showed a weight loss of 65.9% in the 25 - 600 ℃ range, consistent to the expected value of 64.3% for the conversion of Zr-TBB to ZrO_2 (Figure S6).

Dynamic light scattering (DLS) of Zr-TBB revealed a number-averaged size of 117.9 ± 1.4 nm, with a polydispersity index of 0.09 (Figure 1f). Transmission electron microscopy (TEM) imaging (Figure 1a, 1b, S4, S5) revealed spherical to cubic morphology for Zr-TBB with a diameter of approximately 100 nm. High resolution TEM (HR-TEM) imaging gave a lattice spacing of 1.7 nm (Figure 1b) for Zr-TBB while the fast Fourier transform (FFT) patterns (Figure 1b inset) revealed the tetragonal symmetry, consistent with projection down to the crystallographic axis (Figure 1d). Additionally, the UV-vis spectrum of Zr-TBB showed the same number of peaks as H_4TBB, with the appearance of a small TBC Soret peak at ~422 nm due to slight oxidation of TBB (4%) during nMOF synthesis.

Photostability of H_4TBB and Zr-TBB was tested in air-saturated DMF at a 5 μM TBB concentration at 740 nm (100 $mW\cdot cm^{-2}$). After irradiation for 5 minutes, the Q_y peak absorbance of H_4TBB dropped to <4% of the original value, indicating its severe photobleaching (Figure 2a). In contrast, Zr-TBB retained 73% and 65% of the Q_y peak absorbance after light irradiation for15 and 30 minutes, respectively, indicating its much enhanced photostability over H_4TBB. The photodecomposition quantum yield of Zr-TBB ($\Phi_{pd} = 8.14 \times 10^{-4}$) was 14 times lower than that of H_4TBB ($\Phi_{pd} = 1.15 \times 10^{-2}$, Table S2). The improved TBB stability of Zr-TBB can be attributed to the spatial constraint of the nMOF framework which prevents TBB from undergoing structural changes before photooxidation can occur and the site isolation effect of Zr-TBB which prevents TBB ligands from biomolecular decomposition. [33] We found photostability of Zr-TBB and H_4TBB was much improved in oxygen-free condition (Figure S7).

We used high resolution mass spectrometry (HR-MS) to characterize the photobleaching products of Zr-TBB and H_4TBB after 740 nm irradiation (100 $mW\cdot cm^{-2}$) in air-saturated DMF for 4 h. Photo-irradiated Zr-TBB was digested with 10% H_3PO_4 in DMSO before HR-MS analysis. For H_4TBB, the $[H_4TBB+H^+]$ peak at m/Z=795.2 disappeared with the appearance of $[M+H^+]$ at 563.5 assignable to (Z)-4-(2-((5-(4-carboxy-benzoyl)-1H-pyrrol-2-yl)(4-carboxy-phenyl)methylene)-3,4-dihydro-2H-pyrrole-5-carbonyl)benzoic acid (**III**, Scheme 1), a known fragmentation product from bacteriochlorin photobleaching.[34] The fragmentation of H_4TBB during photooxidation was supported by the UV-vis spectrum which showed two new peaks at 327 nm and 406 nm for **III** and disappearance of all peaks corresponding to H_4TBB (Figure S7e). In contrast, only H_4TBC at m/Z=793.3 ($[M+H^+]$) was recovered from the digested photo-irradiated Zr-TBB with no evidence of known photo-fragments. TBC can be generated by direct oxidization the pyrroline ring of TBB without significant structural change on the bacteriochlorin.

UV-vis spectroscopy was used to quantify photobleaching products of H_4TBB (Figure 2c) and Zr-TBB (Figure 2d) after light irradiation for 1-30 minutes (Figure S7a, S7b). H_4TBB was nearly completely photobleached (95%) within 5 minutes to generate mostly fragmentation product **III** (95%) and a negligible amount of H_4TBC (<0.1%). In contrast, Zr-TBB retained 84% TBB in 5 minutes with the formation of 4% TBC. Only 12% of TBB decomposed into unknown photoproducts. As TBC is also a good PS, TBB retained 80% and 74% PDT efficacy after light irradiation for 15 and 30 minutes, respectively. The photostability of Zr-TBB was supported by the maintenance of crystallinity as determined by PXRD (Figure 2b).

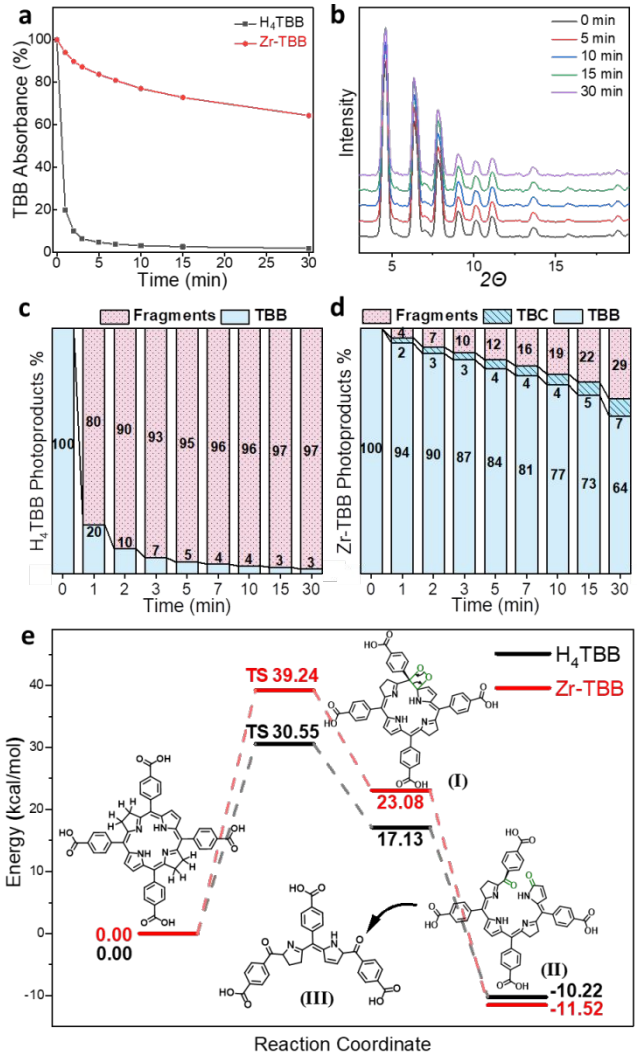


Figure 2. (a) Time-dependent TBB UV-vis absorbance after light irradiation in air-saturated DMF. (b) Time-dependent PXRD patterns of Zr-TBB after light irradiation. Percentages of photoproducts (TBB, TBC, fragments) of H_4TBB (c) and Zr-TBB (d) throughout 30-minute light irradiation. (e) Energy profiles of TBB photo-fragmentation in H_4TBB and Zr-TBB calculated by DFT.

Photobleaching of bacteriochlorins typically starts with [2+2] peroxidation reaction between C=C double bonds and O_2 (Figure 2e).[35] The peroxidized intermediate **I** breaks the π-conjugated bacteriochlorin ring and converts sp^2-carbons into sp^3-carbons, leading to significant distortion from the planar structure of TBB. The peroxide bridge is cleaved into two ketones in intermediate **II** via retro-[2+2] cyclization. Successive peroxidation and retro-[2+2] cyclization form fragmentation product **III**. However, the rigid framework of Zr-TBB prohibits TBB ligands from undergoing large structural changes, shutting down light-mediated peroxidation pathway. The pyrroline rings of the bacteriochlorin can still be oxidized to form TBC ligands without disturbing π-conjugation.

Density functional theory (DFT) calculations were performed to support the photostability difference of bacteriochlorins in H_4TBB and Zr-TBB (Figure 2e). The crystal structure of Zr-TBB was used and the structures of the carboxylate groups were frozen during DFT optimization to mimic spatial constraints in the nMOF. In the calculated energy profiles, H_4TBB displayed a $\Delta G^{\neq}$ of 30.6 kcal/mol (1.33 eV) while the constrained TBB in Zr-TBB exhibited a much higher $\Delta G^{\neq}$ of 39.2 kcal/mol (1.70 eV). The 1.69 eV energy in the 740 nm light source was thus sufficient to overcome the $\Delta G^{\neq}$ in H_4TBB but insufficient to overcome the $\Delta G^{\neq}$ in Zr-TBB, which explained the resistance of Zr-TBB to peroxidation and photo-fragmentation.

Bacteriochlorins can generate multiple ROSs via both type I (O_2^-, H_2O_2, and ·OH) and type II ($^1O_2$) mechanisms.[36] The generation of O_2^-, H_2O_2, ·OH, and $^1O_2$ by H_4TBB and Zr-TBB was confirmed by electron paramagnetic resonance, hydrogen peroxide detection kit, aminophenyl fluoroscein assay (APF), and singlet oxygen sensor green assay (SOSG), respectively. Due to photobleaching, H_4TBB showed much weaker signals of type I ROSs than Zr-TBB (Figure S12-S14). Similarly, $^1O_2$ generation of H_4TBB reached plateau within 1 minute of light irradiation while Zr-TBB showed a linear increase of $^1O_2$ signal throughout the 15-minute experiment (Figure S15).

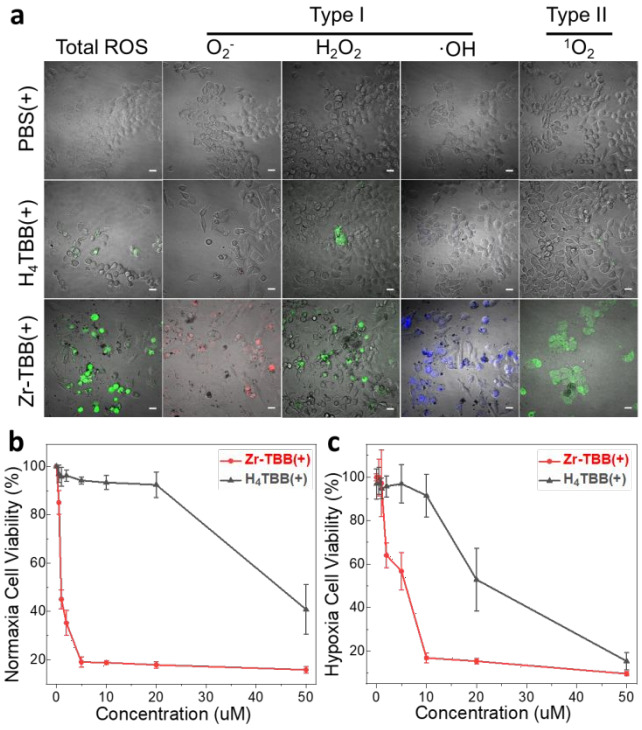


Figure 3. (a) CLSM images of various ROS species generated in 4T1 cells after light irradiation. Total ROS was detected by H2DCFDA assay. Scale bar is 20 μm. (b,c) MTS assays of Zr-TBB(+) and H_4TBB(+) under normoxic (b) and hypoxic (c) conditions.

We next examined cellular uptake, *in vitro* ROS generation, and cytotoxicity of H_4TBB and Zr-TBB on 4T1 murine breast carcinoma cells. ICP-MS and UV-vis studies showed that 4T1 cells uptook significantly more Zr-TBB than H_4TBB (Figure S17). The *in vitro* generation of O_2^-, H_2O_2, ·OH and $^1O_2$ by Zr-TBB plus light irradiation [denoted Zr-TBB(+)] was detected under confocal laser scanning microscopy (CLSM) with superoxide detection, intracellular hydrogen peroxide, coumarin-3-carboxylic acid assay and SOSG assay kits, respectively (Figure 3a). The generation of $^1O_2$ and O_2^- by Zr-TBB(+) was confirmed by flow cytometric analyses. H_4TBB(+) generated much less ROSs than Zr-TBB(+), likely due to low cellular uptake, oxidation, and photobleaching. Zr-TBB(+) efficiently generated four different kinds of ROSs to facilitate type I and type II PDT. The cytotoxicity of Zr-TBB(+) was determined by MTS assay. Under normoxic condition, Zr-TBB(+) exhibited an IC_{50} of 0.91±0.77 μM on 4T1 cells while

H_4TBB(+) did not show any cytotoxicity at ≤20 μM (Figure 3b). Under hypoxic condition, the IC_{50} values of Zr-TBB(+) and H_4TBB(+) on 4T1 cells were 2.94±0.76 μM and 19.50±0.82 μM, respectively (Figure 3c). The increased cytotoxicity of H_4TBB(+) under hypoxia likely resulted from reduced photobleaching at low O_2 concentration. The apoptosis of 4T1 cells after PDT treatments were evaluated by flow cytometry with Annexin-V and propidium iodide staining. Zr-TBB(+) treated cells gave significantly stronger apoptosis signals than those treated with H_4TBB(+) and PBS(+) (Figure S19). Flow cytometry and CLSM imaging studies also showed that Zr-TBB(+) treated cells exhibited much stronger calreticulin (CRT) signals than those treated with H_4TBB(+) and PBS(+) (Figure S23-24), indicating more pronounced immunogenic cell death (ICD) caused by Zr-TBB(+).

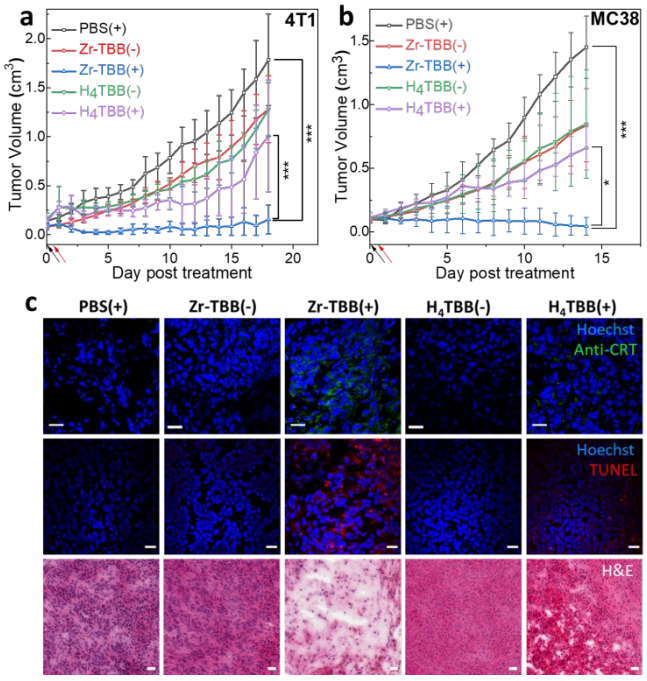


Figure 4. Antitumor efficacy on 4T1 bearing BALB/c mice (a) and MC38 bearing C57Bl/6 mice (b). (c) CLSM imaging of cell surface CRT (top) and cell apoptosis (middle) and H&E staining showing severe apoptosis and necrosis (bottom) after Zr-TBB(+) treatment on 4T1 tumors. Scale bar is 20 μm.

The *in vivo* anti-tumor efficacy was investigated on subcutaneous 4T1 bearing BALB/c mice and murine colon carcinoma MC38 bearing C57Bl/6 mice. Zr-TBB(+) exhibited excellent therapeutic effects to afford 91% tumor growth inhibition and a 40% cure rate on 4T1 model (Figure 4a) and 97% tumor growth inhibition and a 60% cure rate on MC38 model. H&E staining showed severe necrosis in in Zr-TBB(+) treated 4T1 tumors. TUNEL (Figure 4c) and CRT expression (Figure 4d) assays by CLSM showed strong apoptosis and ICD induced by Zr-TBB(+) treatment. Finally, steady body weight and minimal abnormalities of major organ sections indicated that Zr-TBB had no systematic toxicity on BALB/c and C57Bl/6 mice.

In summary, we report the use of the framework of Zr-TBB nMOF to stabilize bacteriochlorins toward oxygen and light irradiation. Zr-TBB mediated effective PDT by generating O_2^-, H_2O_2, ·OH, and $^1O_2$ via both type I and type II mechanisms. Zr-TBB showed superb *in vivo* antitumor efficacy on mouse tumor models of breast and colon cancers to afford cure rates of 40% and 60%, respectively. nMOFs thus present a unique platform to design novel nanophotosensitizers based on bacteriochlorins and other unstable photosensitizing molecules.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Data for Hf-TBB single crystal (CIF)

Synthesis and characterization of H_4TBB and Zr-TBB, ROS generation and mechanism, anticancer efficacy, and DFT calculation (PDF)

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Notes

The authors declare no competing financial interest.

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