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Nanoscale Metal–Organic Layer Isolates Phthalocyanines for Efficient Mitochondria-Targeted Photodynamic Therapy

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Abstract

Zinc-phthalocyanine (ZnPc) photosensitizers (PSs) have shown great potential in photodynamic therapy (PDT) owing to their strong absorption at long wavelengths (650–750 nm), high triplet quantum yields, and biocompatibility. However, the clinical utility of ZnPc PSs is limited by their poor solubility and tendency to aggregate in aqueous environments. Here we report the design of a new nanoscale metal–organic layer (nMOL) assembly, ZnOPPC@nMOL, with ZnOPPC [ZnOPPC = zinc(II)-2,3,9,10,16,17,23,24-octa(4-carboxyphenyl)phthalocyanine] PSs supported on the

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/jacs.0c12330>.

Synthesis and characterization of ZnOPPC, Hf₁₂-Ir nMOL, and ZnOPPC@nMOL, ROS generation, mitochondria targeting properties, and anticancer efficacy studies (PDF)

Movie S1 (MP4)

Movie S2 (MP4)

Movie S3 (MP4)

Movie S4 (MP4)

Movie S5 (MP4)

Movie S6 (MP4)

Movie S7 (MP4)

Movie S8 (MP4)

Movie S9 (MP4)

The authors declare no competing financial interest.

secondary building units (SBUs) of a Hf_{12} nMOL for PDT. Upon irradiation, SBU-bound ZnOPPC PSs absorb 700 nm light and efficiently sensitize the formation of singlet oxygen by preventing aggregation-induced self-quenching of ZnOPPC excited states. With intrinsic mitochondria-targeting capability, ZnOPPC@nMOL showed exceptional PDT efficacy with >99% tumor growth inhibition and 40–60% cure rates on two mouse models of colon cancer.

Photodynamic therapy (PDT) utilizes a photosensitizer (PS), light, and intratumoral oxygen to produce cytotoxic reactive oxygen species (ROS) for antitumor responses.^{1–6} Although PDT can effectively destroy local tumors with minimal systemic toxicity, its clinical utility is limited by the photophysical and pharmacokinetic properties of the PS, photosensitivity of the patient, and short tissue penetration depth of visible light.^{7–10} As highly conjugated analogues of porphyrins, phthalocyanines (PCs) possess several ideal properties as PSs: (1) strong absorption in the therapeutic window of PDT (650–800 nm) for reduced PS doses; (2) high triplet quantum yields and long triplet lifetimes in metalated PCs (M-PCs) for enhanced ROS generation; (3) high stability; (4) biocompatibility and minimal dark toxicity.^{11–13} However, few M-PCs have reached clinical testing for PDT due to their poor solubility and tendency to aggregate in aqueous environments, which induces self-quenching of excited M-PCs and significantly reduces PDT efficacy.^{14–16}

Nanoscale metal–organic frameworks (nMOFs) have shown great potential in biomedical applications.^{17–22} With structural and compositional tunability, crystallinity, framework rigidity, high porosity, and biodegradability, nMOFs have been used to incorporate PSs for PDT.^{23–26} The crystalline and rigid frameworks of nMOFs prevent photobleaching and self-quenching of PSs to significantly increase PDT efficacy.^{27,28} The dimensionality of nMOFs can be reduced to afford nanoscale metal–organic layers (nMOLs) with monolayer thickness, which further enhances ROS diffusion for cytotoxic effects on tumor cells.^{29,30} The therapeutic efficacy of PDT can also be enhanced by targeting PSs to subcellular organelles, such as mitochondria. Generation of cytotoxic ROS in mitochondria disrupts key biological processes such as energy production and activates apoptotic pathways via caspase activation.^{31–33} nMOFs with sufficient lipophilicity and positive charge can target mitochondria^{34–36} without relying on auxiliary triphenylphosphonium moieties.^{37,38}

Herein we report the design of a Hf_{12} -Ir nMOL comprising Hf_{12} secondary building units (SBUs) and $\text{Ir}(\text{DBB})[\text{dF}(\text{CF}_3)\text{-ppy}]_2^+$ ligands [H_2DBB -Ir-F, DBB = 4,4'-di(4-benzoato)-2,2'-bipyridine; $\text{dF}(\text{CF}_3)\text{ppy}$ = 2-(2,4-difluorophenyl)-5-(trifluoromethyl)pyridine] for effective delivery of zinc(II)-2,3,9,10,16,17,23,24-octa(4-carboxyphenyl)phthalocyanine (ZnOPPC) PSs for mitochondria-targeted PDT (Figure 1). ZnOPPC was postsynthetically incorporated onto the nMOL via carboxylate exchange. Upon light irradiation, SBU-bound ZnOPPC PSs absorb light and undergo intersystem crossing to the triplet excited state, which, in the presence of $^3\text{O}_2$, efficiently generates cytotoxic $^1\text{O}_2$.^{39,40} The rigid, ultrathin, and positively charged nMOL isolates ZnOPPC PSs to prevent self-quenching, while allowing efficient ROS diffusion and translocation of the nMOL into mitochondria to afford a highly effective PDT treatment of colorectal cancer.^{35,41–45}

The ZnOPPC ester was synthesized by refluxing 4,5-bis(4-methoxycarbonylphenyl)phthalonitrile, $\text{Zn}(\text{OAc})_2$ and 1,8-diazabicyclo[5.4.0]undec-7-ene in

n-pentanol for 36 h (Figure S4). High-resolution mass spectrometry (HRMS) revealed a mixture of methyl and pentyl esters in the product due to transesterification with *n*-pentanol (Figure S5). Hydrolysis of the ZnOPPC esters yielded the desired acid form of ZnOPPC. HRMS showed a single molecular ion peak with isotopic peaks matching that of ZnOPPC (Figure S8).

The UV–visible spectrum of ZnOPPC in dimethyl sulfoxide (DMSO) exhibited three peaks characteristic of ZnPc (Figure 2a).⁴⁶ The B-band peak at $\lambda_{\text{max}} = 370$ nm is assigned to a π – π^* transition with a molar extinction coefficient (ϵ) of $147\,200\text{ M}^{-1}\text{ cm}^{-1}$, which is 2–3 times lower than those of 5,10,15,20-tetra(*p*-benzoato)porphyrin (H₄TBP)⁴⁷ and 5,10,15,20-tetra(*p*-benzoato)chlorin (H₄TBC).⁴⁸ This difference may alleviate photosensitivity side-effects for ZnOPPC.^{49–51} The Q₀₂-band peak at $\lambda_{\text{max}} = 628$ nm is assigned to an n – π^* transition with $\epsilon = 61\,000\text{ M}^{-1}\text{ cm}^{-1}$, and the Q₀₁-band peak at $\lambda_{\text{max}} = 697$ nm is a π – π^* (HOMO–LUMO) transition with $\epsilon = 343\,600\text{ M}^{-1}\text{ cm}^{-1}$.⁵² The Q₀₁ band has ~72 times larger ϵ value than the longest wavelength Q-band absorption of H₄TBP ($\epsilon_{646} = 4800\text{ M}^{-1}\text{ cm}^{-1}$). Compared to H₄TBP, the Q₀₁-band peak of ZnOPPC at 697 nm not only has a longer absorption wavelength for better tissue penetration but also has a significantly higher ϵ value because PCs lack the near-degeneracy of the a_{1u} and a_{2u} orbitals and mixing of the associated electronic configurations that led to cancellation of the transition dipole moments in porphyrins.⁵³

Hf₁₂-Ir nMOL was synthesized by heating HfCl₄ and H₂DBB-Ir-F in *N,N*-dimethylformamide (DMF) with trifluoro-acetic acid (TFA) and water as modulators at 80 °C (Figure S16).⁵⁴ ZnOPPC@nMOL was synthesized by vigorously stirring a mixture of ZnOPPC and Hf₁₂-Ir nMOL in DMF/ethanol (2:1) at room temperature (Figure S16). The ZnOPPC loading was determined as 14.9 wt % based on inductively coupled plasma-mass spectrometry (ICP-MS) and UV–visible absorption spectroscopy (Figure S9), which corresponds to a 1.1:1 ratio of ZnOPPC:Hf₁₂ SBU. Additionally, the decrease of the TFA peak intensity in the ¹⁹F NMR spectra of digested ZnOPPC@nMOL compared to the bare Hf₁₂-Ir nMOL confirmed the exchange of ZnOPPC with TFA-capping ligands (Figures S18, S26). Based on these results, ZnOPPC@nMOL was formulated as (ZnOPPC)_{1.1}@Hf₁₂(μ_3 -O)₈(μ_3 -OH)₈(μ_2 -OH)₆(DBB-Ir-F)₆(TFA)_{4.9}.

Transmission electron microscopy (TEM) imaging showed that ZnOPPC@nMOL maintained the same monolayer morphology as Hf₁₂-Ir nMOL (Figures 2b and S17). Atomic force microscopy (AFM) supported the monolayer structure of Hf₁₂-Ir and ZnOPPC@nMOL with thicknesses of 1.8 and 3.5 nm, respectively, which are consistent with the modeled heights of Hf₁₂ SBUs capped with TFA ligands (Figure 2c) and ZnOPPC (Figure 2d), respectively. Additionally, the UV–visible spectrum of ZnOPPC@nMOL showed both characteristic peaks for ZnOPPC and Hf₁₂-Ir (Figure S23). Dynamic light scattering (DLS) of ZnOPPC@nMOL revealed a number-averaged size of 156.2 ± 6.4 nm with a polydispersity index of 0.10 (Figure 2e), which is slightly larger than that of Hf₁₂-Ir nMOL at 133.2 ± 8.0 nm. The cationic framework of Hf₁₂-Ir showed a highly positive zeta potential of $+36.3 \pm 0.7$ mV, while ZnOPPC@nMOL showed a negative zeta potential of -19.3 ± 1.0 mV, consistent with installation of negatively charged ZnOPPC (Figure S21). The powder X-ray diffraction (PXRD) pattern of ZnOPPC@nMOL also matched well with

the experimental and simulated PXRD patterns of the bare nMOL (Figure 2f). The stability of ZnOPPc@MOL in phosphate-buffered saline (PBS) was demonstrated by PXRD and DLS after incubation at 37 °C or 700 nm LED irradiation (Figures 2f, S27, S28).

Confocal laser scanning microscopy (CLSM) imaging showed time-dependent enrichment of Hf₁₂-Ir into mitochondria of murine colorectal cancer MC38 cells. Mitochondria were labeled by MitoTracker Red CMXRos, and Hf₁₂-Ir nMOLs were detected by intrinsic fluorescence from DBB-Ir-F. At 15 min after nMOL incubation, most of the Hf₁₂-Ir fluorescence was outside of mitochondria, likely taken up and trapped in endo/lysosomes (Figure S40). From 30 min to 2 h (Figures 3a, S6b, S41, S42, S43), the fluorescence of Hf₁₂-Ir outside of mitochondria decreased, while colocalization between mitochondria and Hf₁₂-Ir signals increased, which was consistent with previous reports of mitochondria targeting by positively charged nanoparticles.^{35,41–45} Surprisingly, we observed the colocalization of ZnOPPc@nMOL with Mito-Tracker 30 min postincubation (Figures S3b, S41, S42, S43), likely due to reversal of surface charge for ZnOPPc@nMOL after protonation of ZnOPPc but not partial release of ZnOPPc (Figure 3d). This hypothesis was supported by a positive zeta potential of $+21.5 \pm 0.3$ mV for ZnOPPc@nMOL measured in a pH = 4 acidic buffer (Figure S22), which mimics the acidic environment of the endo/lysosome. At 2 h, the Pearson's coefficients of colocalization of ZnOPPc@nMOL and Hf₁₂-Ir nMOL with MitoTracker reached 0.838 and 0.979, respectively (Figure S44). Efficient translocation of nMOLs into mitochondria was supported by Hf ICP-MS analysis (Figure S45). Eight hours after PDT treatment of ZnOPPc@ nMOL (100 mW/cm², 10 min; "+" and "-" denote with and without light irradiation, respectively), depolarization of mitochondria membrane potential and release of cytochrome *c* were observed by CLSM (Figures 4a,b, S47), indicating the disruption of mitochondria by PDT treatment with ZnOPPc@ nMOL.

We then examined whether the isolation of ZnOPPc PSs on the nMOL SBUs can facilitate ROS generation and diffusion and enhance cellular uptake to elicit stronger cytotoxic effects. Singlet oxygen sensor green (SOSG) assays revealed that ZnOPPc@nMOL significantly enhanced ¹O₂ generation compared to ZnOPPc in a test tube (Figure 3e) and *in vitro* (Figures 4c, S36, S37), suggesting that conjugation of ZnOPPc PSs on the nMOL reduced aggregation-induced self-quenching. ZnOPPc was sparingly soluble and severely aggregated in serum-containing media (Figure S32). As a result, ZnOPPc@ nMOL showed 12 times higher cellular uptake than ZnOPPc (Figure 3c). Owing to enhanced ¹O₂ generation, high cellular uptake, and strong mitochondria-targeting effect, ZnOPPc@ nMOL(+) was highly cytotoxic with an IC₅₀ of 0.11 μM (Figures 3f, S31). In contrast, ZnOPPc(+) did not inhibit proliferation or cause morphology changes at concentrations up to 10 μM (Figures S31, S32, S33, Movies S1–S9).

PDT-induced apoptosis and immunogenic cell death were evaluated on MC38 cells by flow cytometry and CLSM. Annexin V and propidium iodine (PI) staining revealed ZnOPPc@nMOL(+) had more severe apoptotic cell death than other groups (Figures 4d, S34, S35). The ZnOPPc@ nMOL(+) group also showed stronger surface translocation of calreticulin (Figures 4e, S38, S39), an "eat-me" signal during immunogenic cell death (ICD). Surface exposure of calreticulin can attract myeloid cells for phagocytosis and antigen presentation to facilitate immune responses.

The *in vivo* PDT efficacy of ZnOPPC@nMOL was evaluated on subcutaneous MC38 tumor-bearing C57BL/6 mice and CT26 tumor-bearing BALB/c mice. Hf₁₂-Ir nMOL and ZnOPPC@nMOL were PEGylated before administration. ZnOPPC, Hf₁₂-Ir, and ZnOPPC@nMOL were intravenously injected into mouse tail veins at an equivalent ZnOPPC dose of 0.1 μ mol (equivalent DBB-Ir-F dose of 0.5 μ mol) followed by 700 nm LED irradiation at the tumor site with a total light dose of 90 J/cm² (100 mW, 15 min). The ZnOPPC@nMOL(+) group showed superior anticancer efficacy with 99.1% and 103.8% tumor growth inhibition (TGI) on the MC38 and CT26 model, respectively. Two out of 5 mice and 3 out of 5 mice were tumor free for MC38- and CT26-tumor-bearing mice, respectively (Figures 5a,b, S48, S49). ZnOPPC-(+) moderately inhibited tumor growth with a TGI_{MC38} of 59.6% and TGI_{CT26} of 37.7%, whereas Hf₁₂-Ir(+) had a minimal effect on tumor growth with a statistically insignificant TGI_{MC38} of 35.6% and TGI_{CT26} of 10.0%. TUNEL and CRT staining revealed severe apoptosis and obvious CRT surface translocation in the ZnOPPC@nMOL(+) tumors, respectively (Figures 5c, S59, S60). ZnOPPC@nMOL(+) treatment caused severe necrosis, as shown in the H&E staining of tumor sections (Figure 5c). ZnOPPC@nMOL(+) thus exhibited pronounced antitumor activity and produced strong ICD, leading to excellent PDT efficacy. All treatment groups showed steady trends in mouse body weights (Figures S51, S56) and minimal aberration in major organ sections (Figure S55), indicating lack of general toxicity. Thus, Hf₁₂-Ir nMOL is a biocompatible and efficient nanodelivery platform for PSs with significantly enhanced therapeutic effects.

In summary, we report the isolation of ZnOPPC PSs on the SBUs of cationic Hf₁₂-Ir nMOLs to prevent aggregation-induced self-quenching of the excited PSs and to target mitochondria. As a result, ZnOPPC@nMOL showed significantly enhanced ¹O₂ generation and superb anticancer efficacy over ZnOPPC. This work demonstrates the potential of using nMOLs to deliver highly potent PSs with nonideal physicochemical and pharmacokinetic properties for effective PDT treatment of cancers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(a) Scheme showing the structure of ZnOPPC@nMOL and efficient $^1\text{O}_2$ generation from isolated ZnOPPC PSs on the SBUs. (b) Schematic showing π - π stacking of two ZnOPPC PSs in solution leading to self-quenching of excited states.

Figure 2.

(a) UV–visible absorption spectra of ZnOPPC and ZnOPPC@nMOL in DMSO showing characteristic B- and Q-band peaks. (b) TEM image of ZnOPPC@nMOL. AFM topography, height profile (inset), and modeled heights of (c) Hf₁₂-Ir nMOL and (d) ZnOPPC@nMOL. (e) Number-averaged diameters in ethanol and (f) PXRD patterns of Hf₁₂-Ir nMOL, as-synthesized ZnOPPC@nMOL, and ZnOPPC@nMOL after soaking in PBS for 16 h, along with the simulated pattern for the Hf₁₂ MOL.

Figure 3.

Hf₁₂-Ir nMOL (a) and ZnOPPc@nMOL (b) colocalized with mitochondria as visualized by CLSM after a 2 h incubation with MC38 cells (scale bars equal 20 μm). (c) Cellular uptake quantified by UV-visible spectroscopy, *N* = 3. (d) Release profile of ZnOPPc@ nMOL in PBS, *N* = 3. (e) ¹O₂ generation of ZnOPPc and ZnOPPc@ nMOL detected by SOSG assay. (f) MTS assay of PDT treatment with ZnOPPc or ZnOPPc@nMOL on MC38 cells, *N* = 6.

Figure 4.

(a) Loss of mitochondria membrane potential by the JC-1 assay. Red channel indicates J-aggregate and green indicates monomer form of JC-1 molecules. (b) Release of cytochrome *c* (green) from mitochondria (red). (c) ¹O₂ generation by the SOSG assay (green). (d) Cell apoptosis visualized by annexin-V (green) and PI (red) staining. (e) ICD detected by CRT expression on the cell surface (green). Cell nuclei were stained by Hoechst 33342 (blue) in (d) and (e). MC38 cells were used, and all scale bars equal 20 μm.

Figure 5. PDT anticancer efficacy on subcutaneous MC38 tumor-bearing C57BL/6 mice (a) and CT26 tumor-bearing BALB/c mice (b), $N=5$. (c) H&E staining (top), TUNEL assay (middle), and CRT staining of excised tumors from treated C57BL/6 mice. Scale bars are 50 μm for H&E staining and 20 μm for the others. * $P < 0.05$ and *** $P < 0.001$ by ANOVA test.