ChemComm

COMMUNICATION

View Article Online
View Journal | View Issue

Cite this: *Chem. Commun.*, 2021, **57**, 3127

Received 3rd February 2021,
Accepted 12th February 2021

DOI: 10.1039/d1cc00645b

rsc.li/chemcomm

A 2-pyridone modified zinc phthalocyanine with three-in-one multiple functions for photodynamic therapy†

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A 2-pyridone modified zinc phthalocyanine (denoted ZnPc-PYR) achieves a one stone for three birds outcome in the photodynamic therapy (PDT) treatment of cancer. ZnPc-PYR can be excited by both 665 and 808 nm light to treat superficial and deep tumors, store and slowly release singlet oxygen ($^1O_2$) to improve its utilization and downregulate the HIF-1 (hypoxia-inducible factor 1) expression level to enhance the tumor cell's sensitivity to PDT treatment under hypoxic conditions.

The mechanical research and clinical application of PDT for cancer treatment have been tremendously developed in recent years. The PDT mechanism refers to a process in which a photosensitizer (PS) accumulated in the tumor tissue is excited by laser irradiation of a specific wavelength. The excited photosensitizer transfers energy to the surrounding O_2 to generate highly toxic reactive oxygen species (ROS) to damage the tumor tissue. Light, PS, and O_2 are the three critical elements in PDT.[1,2] The ROS generation capacity and utilization efficiency are essential indicators to evaluate the PDT activity. It has been found that the insufficient penetration depth of the light,[3,4] the tumor hypoxic microenvironment,[5,6] and the short lifespan of some ROS, particularly, $^1O_2$,[7] restrict the PDT performance.

The excitation wavelength of classic PSs is in the range of visible light, for which the tissue penetration depth is insufficient. Also,[8] hemoglobin and melanin in tissues have strong absorption of light from 600 to 700 nm, inducing a reduction in the transfer efficiency of light. All of the above problems will lead to an insufficient effect of PDT in treating deep lesions. In contrast, the penetration depth and transfer efficiency of near-infrared (NIR) light are efficient for deep-site lesion treatment. Therefore, PSs that can be excited by both visible and NIR light could effectively treat superficial and deep tumors.

$^1O_2$ is the primary ROS for most of the PSs in PDT. However, its lifespan is incredibly short (<40 μs).[7,9,10] Thus, many $^1O_2$ are quenched before oxidative damage to tumor cells. Therefore, the inadequate utilization of $^1O_2$ would significantly reduce the therapeutic effect of PDT.

Besides, hypoxia is a unique characteristic of the tumor microenvironment. The PDT process consumes O_2 to aggravate the tumor hypoxia further and eventually lead to a poor PDT outcome. Moreover, under hypoxic conditions, HIF-1 is up-regulated. HIF-1 upregulation is a predominant event in the promotion of cancer progression, such as angiogenesis, metastasis, and apoptosis resistance.[11,12] HIF-1 up-regulation could enhance the cells' resistance to various cancer treatments, including PDT. The HIF-1 expression level is positively correlated with patient mortality and treatment tolerance. Thus, HIF-1 inhibition can enhance the cancer cell's sensitivity to PDT to enhance the treatment efficiency under hypoxic conditions.[13,14]

ZnPc is a representative of a third-generation PS. Based on the above three bottlenecks in PDT treatment, we designed and synthesized a 2-pyridone modified ZnPc (denoted ZnPc-PYR) with multiple functions (Fig. 1 and Fig. S1, S3, ESI†).

First, the ZnPc-PYR can be excited by both 665 and 808 nm light to generate ROS. Compared with the 665 nm light, the 808 nm light has a deep penetration depth in tumor tissue. Therefore, the ZnPc-PYR could achieve high-efficiency PDT treatment of superficial and deep tumors, simultaneously. Second, the ZnPc-PYR was modified by four 2-pyridones. $^1O_2$ can oxidize 2-pyridone to its internal peroxide to store $^1O_2$. Then, the stored $^1O_2$ was gradually released under 37 °C to improve the utilization of $^1O_2$. Third, the ZnPc-PYR can significantly down-regulate the HIF-1 level to enhance the tumor cell's sensitivity to PDT treatment under hypoxic conditions.[15–17] Our research indicated that ZnPc-PYR exhibited a satisfactory PDT outcome for cancer

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† Electronic supplementary information (ESI) available: Materials and methods, the total ROS and $^1O_2$ generation of ICG, some *in vitro* toxicity data and *in vivo* biocompatibility. See DOI: 10.1039/d1cc00645b

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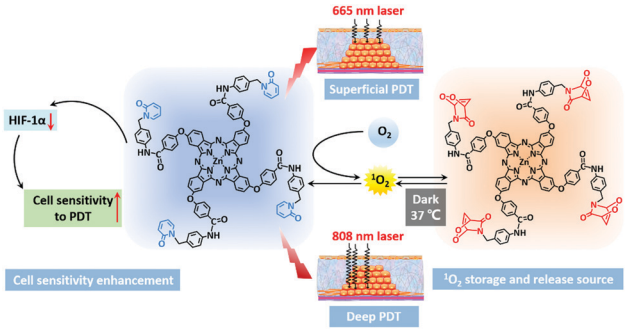

Fig. 1 Schematic diagram of ZnPc-PYR with three-in-one multiple functions for tumor PDT.

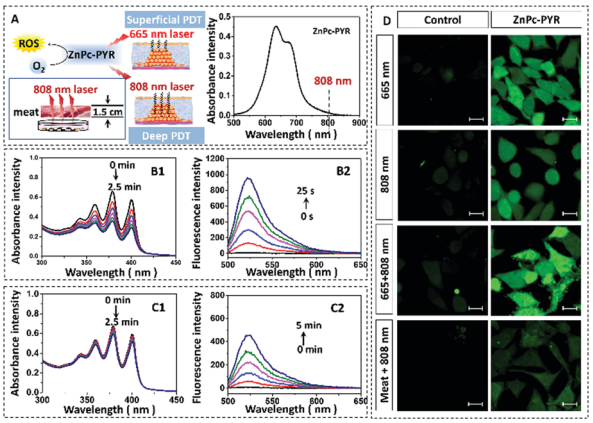

Fig. 2 (A) Absorbance spectra of ZnPc-PYR and the schematic presentation of its potential application in the superficial and deep tumor PDT treatment. (B) The $^1O_2$ (1) and total ROS (2) generation in aqueous solution of ZnPc-PYR post 665 nm light irradiation. (C) The $^1O_2$ (1) and total ROS (2) generation in aqueous solution of ZnPc-PYR post 808 nm light irradiation. (D) *In vitro* ROS generation of ZnPc-PYR under various light irradiation conditions (bar = 20 μm).

treatment. The above positive results provided a reference for research on the design and synthesis of multiple function integrated PSs against the bottleneck of PDT treatment.

The water solubility of ZnPc-PYR is low. Thus, the stock solution of ZnPc-PYR was prepared in DMSO. After addition to an aqueous solution, ZnPc-PYR tends to aggregate leading to precipitation. CrEL (Cremophor EL) is a non-ionic surfactant with excellent solubility and emulsifying activity. CrEL is widely used to improve drug solubility since it is a nontoxic and nonirritating substance in various acute toxicity and chronic toxicity tests.[18,19] Thus, CrEL was used as a solubilizer of ZnPc-PYR to improve its water dispersion ability and activity (Scheme S1 and Fig. S4, S5, ESI†). And in all of the chemical and activity studies for ZnPc-PYR, 0.001% CrEL was added.

The classic ZnPcs are activated by red light, which has a limited tissue-penetration depth and is suitable to treat superficial cancer. In contrast, NIR (700–1000 nm) has greater penetration depths than red light and is suitable to treat large and deep-seated tumors.[20,21]

As shown in Fig. 2A, ZnPc-PYR has strong absorbance around 665 nm, indicating that it can be excited by red light to generate ROS for superficial cancer treatment. Interestingly, ZnPc-PYR also has tail absorbance around 800 nm. We proposed that it could be excited by NIR light to generate ROS for deep tumor treatment. To verify this hypothesis, we detected the ROS generation ability of ZnPc-PYR under 665 and 808 nm light irradiation using ADPA (a disodium salt of 9, 10-anthracenedipropionic acid) and DCFH (dichlorofluorescin diacetate) as a $^1O_2$ and total ROS probe, respectively.

As shown in Fig. 2B and C, obviously $^1O_2$ and total ROS generation were detected by ZnPc-PYR post 665 and 808 nm light irradiation. The ZnPc-PYR absorbance intensity at 808 nm is not strong enough. So, we worry that its NIR light-triggered PDT activity may not be strong enough. To solve this concern, we compared the $^1O_2$ and total ROS generation ability between ZnPc-PYR and ICG (Indocyanine Green), a classic NIR-I light-triggered PS and photothermal agent, under 808 nm light irradiation.[22,23] As shown in Fig. S6 (ESI†), the results indicated that these abilities of ZnPc-PYR were higher than those of ICG. Tail absorbance[2] or fluorescence is widely used for cancer treatment or diagnosis. For example, the NIR-I (700–1000 nm) fluorescent ICG has been approved by the United States Food and Drug Administration (FDA) for NIR-II (1000–1700 nm) diagnosis using its tail fluorescence in the NIR-II region.[24]

We proved that ZnPc-PYR in the presence of CrEL is taken up by cells through endocytosis mediated by caveolae and micropinocytosis (Fig. S7, ESI†). The *in vitro* ROS generation ability of ZnPc-PYR was studied using DCFH-DA as a probe. Similarly, ZnPc-PYR can generate ROS effectively inside cancer cells post irradiation by 665 and 808 nm light. Besides, the total ROS level in 665 + 808 nm light irradiation cells was significantly higher than that of 665 or 808 nm alone treated cells (Fig. 2D). Notably, 808 nm light can penetrate 1.5 cm pork to trigger ZnPc-PYR to generate ROS inside cancer cells. Therefore, ZnPc-PYR can be used in a dual-wavelength treatment for superficial and deep tumors.

The above results showed that the ZnPc-PYR could generate adequate $^1O_2$ under 665 or 808 nm light irradiation. $^1O_2$ is the primary cytotoxic ROS in PDT. However, its lifetime is very short (<40 ns).[7] Therefore, many $^1O_2$ would be quenched before damaging the target biomolecules, resulting in low utilization of $^1O_2$, limiting the PDT efficiency. 2-Pyridone can be oxidized by $^1O_2$ leading to endoperoxide formation, which could gradually release $^1O_2$ under 37 °C. Thus, ZnPc-PYR should have $^1O_2$ storage and gradual release functions to make full use of $^1O_2$ (Fig. S8A, ESI†).[25,26] Moreover, in order to verify that the 2-pyridone modifier is critical to the storage and release of $^1O_2$, we synthesized a phenyl-modified ZnPc (ZnPc-BZA, Fig. S2, ESI†) as a negative control.

The $^1O_2$ storage and release function of ZnPc-PYR was studied by monitoring the oxidation-caused absorption decrease of ADPA in aqueous solution. As shown in Fig. S9 and S10 (ESI†), for ZnPc-BZA, the absorption peak of ADPA at 378 nm (λ_{max} of ADPA) decrease can only be detected under the light irradiation cycle. In the dark incubation cycle (37 °C) of ZnPc-BZA, the absorption intensity decrease was not observed, but an increase in the absorption value was observed. ADPA

can be oxidized by $^1O_2$ to its endoperoxide formation to store $^1O_2$ and induce its absorbance to decrease. The $^1O_2$ release from endoperoxide formation to revert to ADPA induced the above absorption enhancement phenomenon. However, the released $^1O_2$ from the endoperoxide formation is not enough to oxidize ADPA. In contrast, for ZnPc-PYR, the decrease of the absorption peak of ADPA at 378 nm can be detected under a light irradiation cycle (665 nm light irradiation) and dark incubation cycle. These results indicated that the ZnPc-PYR possesses an efficient $^1O_2$ storage and release function, and the 2-pyridone modification is critical for this function.

The *in vitro* $^1O_2$ generation detection further proved the $^1O_2$ storage and release function of ZnPc-PYR, both under 665 and 808 nm light irradiation. The *in vitro* $^1O_2$ generation was detected using SOSG as a probe using CLSM and FCM technology. As shown in Fig. S8B (ESI†), a large amount of $^1O_2$ was generated immediately in cancer cells by ZnPc-PYR after irradiation by 665 or 808 nm light. After all the cells had been continuously incubated in the incubator (37 °C) for 6 hours in the dark, the cells continued to show enhancement of the fluorescence intensity, indicating that $^1O_2$ can be continuously released in these cells.

Hypoxia is a primary characteristic of the solid tumor microenvironment. The PDT process consumes O_2 to aggravate tumor hypoxia.[27] HIF-1 is composed of a constitutively expressed subunit (HIF-1β) and an O_2-regulated subunit (HIF-1α). The up-regulation of HIF-1 is a predominant event in cancer progression promotion, such as angiogenesis, metastasis, and apoptosis resistance. More significantly, HIF-1 upregulation is associated with PDT treatment resistance. Thus, HIF-1 inhibition is an effective way to enhance the PDT treatment efficiency.[13,14]

As shown in Fig. 3A, after treating with ZnPc-PYR, the expression level of HIF-1α in cancer cells was significantly down-regulated. In contrast, DMSO or CrEL did not influence HIF-1α expression. The above positive results implied that ZnPc-PYR could enhance cancer cell sensitivity to PDT treatment and possess satisfactory PDT activity under hypoxic conditions.

ICG has a maximum absorption band of around 800 nm. In contrast, just as mentioned above, the ZnPc-PYR absorbance intensity at 808 nm is not strong enough. To verify that the NIR light-triggered PDT activity of ZnPc-PYR is sufficient, we compared the 808 nm light-triggered cancer cell killing ability between ICG and ZnPc-PYR. As shown in Fig. S11 (ESI†), post 808 nm light irradiation, although the cancer cell killing ability of ZnPc-PYR is lower than that of ICG, about 50% of cancer cells were killed in the ZnPc-PYR treated group. Besides ROS, ICG could effectively absorb NIR light and convert it into heat to achieve photothermal therapy (PTT).[28] ZnPc-PYR did not have a photothermal conversion capability. Besides, pretreating by ascorbic acid, an antioxidant regent, can inhibit the cancer cell killing activity of ZnPc-PYR post 808 nm light irradiation. Thus, the cancer cell killing mechanism by ZnPc-PYR post 808 nm light irradiation is a PDT process but not a PDT-PTT combination process (Fig. S12, ESI†).

We evaluated the dual-wavelength triggered *in vitro* PDT effect. The *in vitro* cancer cell killing capability was studied by

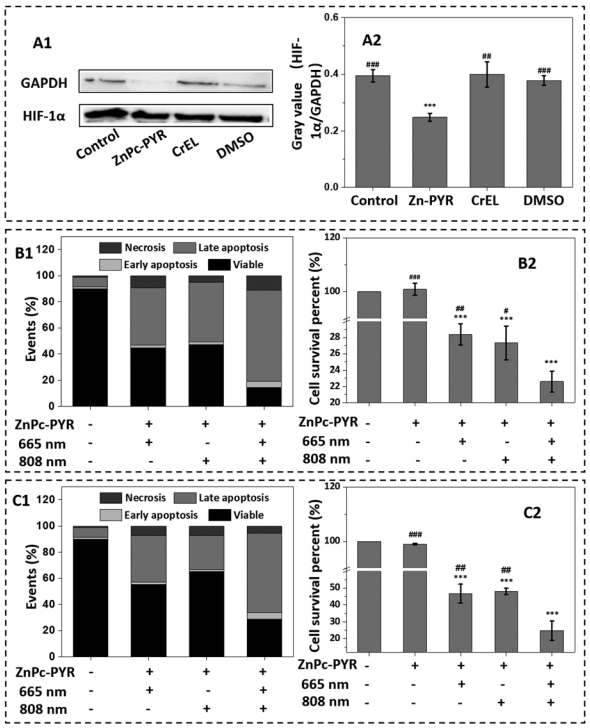

Fig. 3 (A) The expression (1) and quantitative comparison (2) of HIF-1α by the western blot method post various treatments. The quantitative analysis of apoptotic cells in various stages (1), and cancer cell killing capability (2), of ZnPc-PYR post various kinds of light irradiation treatments under normoxic (B) and hypoxic (C) conditions in HeLa cells. (Data are expressed as means ± SD; $^*P < 0.05$; $^{**}P < 0.01$ $^{***}P < 0.001$ *versus* control; $^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$ *versus* ZnPc-PYR + 665 + 808 nm group.)

MTT assay. The PDT induced cell apoptosis was studied using a PI/Annexin IV-FITC kit by FCM. The dark cytotoxicity test of ZnPc-PYR, CrEL and ICG indicated that they do not have dark toxicity under our experiment conditions (Fig. S13, ESI†). As shown in Fig. 3B and Fig. S14A, S15 (ESI†), under normoxic conditions, ZnPc-PYR has 665 and 808 nm light triggering PDT activity to induce significant cancer cell apoptosis and cell death. Moreover, the *in vitro* PDT activity of the dual-light treatment (665 nm + 808 nm) group was higher than that of the single-light treatment groups. The *in vitro* photodynamic activities of ZnPc-PYR were also examined in the presence of 1.5 cm pork to prove its deep tumor treatment potential (Fig. S16, ESI†).

Moreover, just as mentioned above, the HIF-1 downregulation function of ZnPc-PYR could enhance the cancer cell sensitivity to PDT treatment under hypoxic conditions. The results in Fig. 3C and Fig. S14B (ESI†) verified this hypothesis. Under the hypoxic conditions, ZnPc-PYR also has satisfactory dual-light induced PDT activity in HeLa cells. The same experiment was also implemented on 4T1 cells, and similar results were obtained (Fig. S17, ESI†). This property is significant for its *in vivo* PDT treatment since the hypoxic and normoxic areas coexist in the solid tumor.

Based on the above positive *in vitro* results, we proposed that ZnPc-PYR would have satisfactory tumor suppression capability

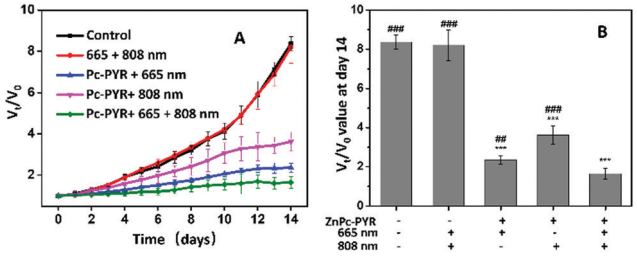

Fig. 4 (A) Tumor volume changes post 14 days of various treatment. (B) The final tumor volume comparison of various groups. (Data are expressed as means ± SD; $^{*}P < 0.05$; $^{**}P < 0.01$ $^{***}P < 0.001$ *versus* control; $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ $^{\#\#\#}P < 0.001$ *versus* ZnPc-PYR + 665 + 808 nm group.)

in vivo. As shown in Fig. S18 (ESI†), no noticeable weight loss or main organ damage (from the H&E staining results of the tissue sections) were detected during 14 days of ZnPc-PYR treatment under various light irradiation conditions, indicating its ideal biocompatibility. During the 14 days of treatment, the tumor volumes of various groups were recorded and compared. As shown in Fig. 4A and B, 665 and 808 nm light irradiation at the tumor tissue did not induce tumor suppression, indicating that the light dose used in this experiment is safe.

In contrast, both 665 and 808 nm light irradiation at the tumor tissue can suppress tumor volume growth. Moreover, the combination treatment of the dual-light group has the best tumor suppression efficiency. Besides, the lesion damage degree of the tumors in various groups was observed by H&E staining (Fig. S18, ESI†). As expected, significant nuclear deformation and shrinkage were detected in the ZnPc-PYR + 665 nm + 808 nm group. Overall, ZnPc-PYR had good biocompatibility and showed excellent *in vivo* PDT activity. The *in vivo* photodynamic activities of ZnPc-PYR were also examined in the presence of 1.5 cm pork to prove its deep tumor treatment potential (Fig. S19, ESI†).

In conclusion, to overcome the problems of insufficient tissue penetration depth of light, limited utilization of $^1O_2$, and hypoxic microenvironment caused cell resistance to the PDT treatment, we synthesized 2-pyridone modified ZnPc (ZnPc-PYR) in this paper. The *in vitro* and *in vitro* experiment results showed that ZnPc-PYR could be excited by the dual wavelengths of 665 and 808 nm to generate massive ROS to achieve superficial and deep tumor PDT treatment. The 2-pyridone in ZnPc-PYR can be oxidized to store $^1O_2$, which could be gradually released under 37 °C to improve the $^1O_2$ utilization. Finally, we also found that ZnPc-PYR can significantly down-regulate HIF-1α expression to enhance the sensitivity of tumor cells to the PDT treatment under hypoxic conditions. Both the MTT experiment and the apoptosis experiment showed that the PDT process of ZnPc-PYR significantly damages tumor cells even under 1% O_2 hypoxic conditions. Therefore, ZnPc-PYR can achieve efficient treatment of tumors based on the above multiple functions.

Natural Science Foundation of China (grant number 21671105), the project (grant number BK20161554) supported by NSF of Jiangsu Province of China, the Priority Academic Program Development of Jiangsu Higher Education Institutions, and the Foundation of Jiangsu Collaborative Innovation Centre of Biomedical Functional Materials (grant number 161090H001) are acknowledged.

Conflicts of interest

There are no conflicts to declare.

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