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Conjugates of folic acids with zinc aminophthalocyanine for cancer cell targeting and photodynamic therapy by one-photon and two-photon excitations

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To improve the photodynamic detection and therapy of cancers (PDT), the folic acid (FA) was conjugated with zinc tetraaminophthalocyanine (ZnaPc) to form ZnaPc-FA. The uptake efficiency of ZnaPc-FA to a FR-positive (folate receptor overexpressed) KB cell line (human nasopharyngeal epidermal carcinoma) was much higher than that of ZnaPc demonstrating an enhanced binding ability of ZnaPc-FA to KB cells. When KB cells were pretreated with free FA and followed by the incubation of ZnaPc-FA, the high uptake rate of ZnaPc-FA disappeared which exhibited the special binding function of the FA terminal of ZnaPc-FA on KB cells. The confocal fluorescence images further showed that the affinity of ZnaPc-FA to FR-negative A549 cells (human lung epithelial carcinoma cancer cells) was very low, confirming that ZnaPc-FA can only target FR-positive cancers. The two-photon absorption cross section of ZnaPc-FA was also higher than that of sulfonated aluminum phthalocyanine (AlPcS), an approved PS for clinical applications. With a 780 nm femto-second (fs)

laser, the fluorescence image of ZnaPc-FA in KB cells under two-photon excitation (TPE) can be clearly seen, and the two-photon induced singlet oxygen in ZnaPc-FA solution was found to be proportional to the irradiation dose of fs laser. The PDT damaging effect of ZnaPc-FA on KB cells was much effective relative to AlPcS under common one-photon excitation, and the killing efficacy of ZnaPc-FA under TPE was 10-fold higher than that of AlPcS. These results suggest that the ZnaPc-FA is a promising candidate for PDT improvements and particularly for TPE PDT.

INTRODUCTION

Photodynamic therapy (PDT) is a new therapeutic modality approved for clinical treatments of several types of cancer and non-oncological disorders.[1-3] The principle of PDT is that the photosensitizer (PS) accumulates in malignant tissues[2], and the subsequent activation of the PS by the light with appropriate wavelength generates singlet oxygen ($^1O_2$) to destroy the lesion. PDT has drawn considerable attention due to its non-invasive treatment nature.[2] However, the PSs have no affinity to cancer cells and their existence in tumors is due to the passive accumulation. The side effect is thus induced as the PSs also extensively exist in skin tissues resulting in a so-called phototoxicity on skin which seriously limits the PDT applications. Therefore, developing a PS with a high affinity to cancers is still a challenge.

It has been found that many cancer lines can be specifically targeted by some special agents. For instance, the Herceptin (HER) (a monoclonal antibody) can target breast cancer cells because the breast cancer cells express a highly specific tumor

associated antigens.[4] The folic acid (FA) has been demonstrated as another effective tumor targeting agent.[5] FA is a low molecular weight pterin-based vitamin required by eukaryotic cells for one-carbon metabolism and de novo nucleotide synthesis.[6, 7] The FA receptor (FR) is a high-affinity membrane protein that is overexpressed in a wide variety of human tumors, including more than 90% of ovarian carcinomas.[8-10] Additionally, the expression of FR in normal tissues is extremely low [11], making FA a useful marker for targeted drug delivery to tumors.[12] It has been shown that the derivative of folic acid still retains strong affinity toward its receptor when the conjugates of FA and drugs are formed via its γ-carboxyl group.[13] The FA has been suggested for the selective delivery of imaging and therapeutic agents to tumor tissues.[9] Particularly, many works used FA contained nanocomposition such as nanoparticles to target cancers for detections and therapies.[14] Nanoparticles have the advantages including the high photoluminescence yield for tumor detection or photo-thermal effect for photo-thermal therapy. However, the toxicity of nanoparticules themselves and their long-term fate in living body due to their low biocompatibility are greatly concerned.

In PDT studies, the commonly used PSs including the porphyrin and phthalocyanine derivatives have been convinced to be safe except the phototoxicity to skins. Directly connecting the PSs with FA is therefore a simply strategy for improving the tumor selectivity of PSs, and so far few works have been reported along this consideration. In this work, Zinc tetraaminophthalocyanine (ZnaPc) was used to conjugate with FA via its γ-carboxyl group forming ZnaPc-FA. The specific

affinity of ZnaPc-FA to FR overexpressed cancer cells was demonstrated by a pair of cell lines of FR-positive KB cells (human nasopharyngeal epidermal carcinoma cell lines) and FR-negative A549 cells (human lung epithelial carcinoma cancer cell lines). The in vitro PDT effect of ZnaPc-FA was further found to be much better than that of sulfonated aluminum phthalocyanine (AlPcS), an approved PS for clinic applications, under common one-photon excitation (OPE) of red light. Recently, a new PDT modality with two-photon excitation (TPE) of near-infrared (NIR) laser pulses has been proposed[15], because the NIR lights have the best tissue penetration depth benefiting the PDT treatments. The two-photon absorption cross section (TPACS) of ZnaPc-FA was found to be higher than that of AlPcS. The in vitro PDT damaging efficiency of ZnaPc-FA was 10-fold high relative to AlPcS under TPE of 780 nm femto-second (fs) laser. These results demonstrate that ZnaPc-FA is a potential PS to improve the FR-positive cancer targeting as well as PDT efficacy particularly under TPE of NIR fs lasers.

RESULTS AND DISCUSSION

The ZnaPc-FA

With the help of dicylcohexylcarbodiimide (DCC), a covalent linkage between the ZnaPc and FA was achieved to form ZnaPc-FA (**Figure 1A**).[13] The absorption and fluorescence spectra of ZnaPc-FA were shown in **Figure 1B**. The as-prepared ZnaPc-FA can dissolve in aqueous solution, but their solubility in pure water is not so good. In aqueous solution containing 1% volume DMSO, the ZnaPc-FA was totally dissolved. Therefore, the ZnaPc-FA can be used for further in vitro experiments, since

1% volume DMSO would not harm culture cells. The aggregation has been found as a big problem for ZnPc[16], because the aggregates of ZnPc lose their photosensitization ability.[17] The absorption spectrum can reveal the aggregation trace for ZnPC compounds as the monomers have the peak at 700 nm whereas the dimmer's band locates around 660 nm.[18] To find out the aggregation states of ZnaPc-FA in solutions, the absorption spectra of ZnaPc-FA in aqueous solution containing 1% DMSO and pure DMSO solution were comparatively measured as shown in **Figure 2A**. In the pure DMSO solution, ZnaPc-FA demonstrated the typical monomer feature with the dominated 700 nm absorption. While in the aqueous solution with 1% DMSO, the 660 nm absorption peak became pronounced, reflecting that the dimmers of ZnaPc-FA extensively existed.[18] If there are no enough monomers existed in the system, the PDT efficiency of ZnaPc-FA will be very low.[19] However, it has been reported that many proteins under physiological conditions can disassociate the aggregates of PC-like compounds[20], which may remain the hope of ZnaPc-FA as the potential candidate of new PS. When the FBS (fetal beef serum) was added into the aqueous solution of ZnaPc-FA, the monomer component increased as the increment at 700 nm absorption band can be seen (**Figure 2B**). To quantitatively measure the monomer percentage in aqueous solutions, the fluorescence comparison method was used based on the fact that the monomers emit fluorescence whereas the emission of dimers can be neglected. As shown in **Figure 2C**, the fluorescence of ZnaPc-FA in aqueous solution (1% DMSO) increased obviously when FBS with different percentages was added, respectively. Comparing the fluorescence intensity with that of monomers (ZnaPc-FA

in pure DMSO), the monomer percentage of ZnaPc-FA in aqueous solution (1% DMSO) after 10% FBS adding was estimated about 25%. With the addition of FBS, the fluorescence peak of ZnaPc-FA was "blue-shift", which is consistent with the previously reported work, owning to the interaction between the ZnaPc-FA and proteins.[21] The considerable percentage of monomers of ZnaPc-FA in serum protein contained systems encouraged us to continue the consequent in vitro works, because there are too many proteins existed in the cytoplasm of cells and some of them are similar to serum proteins in both structure and function. The cytoplasm proteins can be expected to interact as FBS to disaggregate the dimmers of ZnaPc-FA into the monomers when ZnaPc-FA has penetrated into cells.

ZnaPc-FA targeted FR-positive KB cells

The cell uptakes of the ZnaPc and ZnaPc-FA in KB cells were measured as the first step to study the specific binding of ZnaPc-FA to FR-positive cells. The same incubation concentration of 1 μM for this pair compounds in aqueous solution (1% DMSO) was selected to incubate KB cells for 50 min. Then the fluorescence images of these incubated cells were acquired by a detection channel with the band-pass filter of (650-730 nm) in a confocal laser scanning microscope (CLSM) under the OPE of 360 nm. As shown in **Figure 3**, the fluorescence image of ZnaPc incubated cells is very weak whereas that of ZnaPc-FA incubated cells is much bright, demonstrating that much more ZnaPc-FA entered KB cells as compared to ZnaPc and reflecting an enhanced binding ability of ZnaPc-FA to KB cells. The strong fluorescence of cellular ZnaPc-FA in **Figure 3B** must come from the monomers suggesting that the ZnaPc-FA have been disaggregated by some proteins in cytoplasm when they penetrated into cells.

The second step was to check that the association of ZnaPc-FA to KB cells was whether FR mediated. If the FR proteins on the surfaces of KB cells were fully occupied by the free FA, the association of ZnaPc-FA would be then blocked. We pretreated KB cells with excess free FA (20 μM) and these free FA treated cells were consequently incubated with ZnaPc-FA (1 μM) for the same 50 min. The very weak fluorescence image of these double-treated cells as shown in **Figure 3C** supports that the association of ZnaPc-FA with KB cells is mediated by the binding of FA terminal of ZnaPc-FA with FR on the surfaces of KB cells.[22]

To convince that the ZnaPc-FA only have the affinity to FR-positive cancer cells but not to the FR-negative cells. The binding of ZnaPc-FA to FR-negative A549 cells was tested under the same conditions. After incubation of ZnaPc-FA (1 μM) for 50 min, the fluorescence image of A549 cells was acquired and shown in **Figure 3D**. The very dim image of A549 cells, in contrast to that of KB cells, shows that the binding efficiency of ZnaPc-FA to A549 cells is very low. These results conclude that the ZnaPc-FA can effectively target FR-positive cancer cells via FR mediated process.

Two photo-absorbance cross section measurements of ZnaPc-FA

The NIR light region is the tissue window, so that the PDT with the NIR lights, particularly for TPE, has become a hot topic in PDT studies.[23] The potential of ZnaPc-FA for TPE PDT with NIR fs lasers was also an interesting issue.[24] For TPE PDT the TPACS of PS is the key factor. We used TPE induced fluorescence to measure the TPACS of ZnaPc-FA by the method of fluorescence comparison with the known TPACS of the reference sample. Differing to the linear process, the TPE induced fluorescence of the sample has the characteristic of excitation power square dependence. **Figure 4** shows the relationship of fluorescence intensities of ZnaPc-FA

with the fs laser power at different wavelengths. The excitation power square dependent results of fluorescence intensity in **Figure 4** exhibit the TPE process of ZnaPc-FA under fs laser irradiations. Taking Rhodamine B as the reference sample with the known TPACS of 210 GM (1GM = 10^{-50} cm^4 s/photon)[25] and comparing their fluorescence intensities under TPE of fs lasers, the TPACS of ZnaPc-FA at different wavelengths were obtained and shown in Table 1. For comparison, the TPACS of AlPcS in water were also measured since AlPcSs exist in monomer form when they dissolve in water. For ZnaPc-FA, the solution was the pure DMSO, because we want to measure the TPACS of the monomers of ZnaPc-FA and only the monomers have the PDT effect. The TPACS of ZnaPc-FA monomers is obviously higher than that of AlPcS monomers, which is consistent with the previous report on Zinc-porphyrin conjugates as they found that the conjugation strength in these asymmetry structure molecules plays an important role on their TPACS.[26] When ZnaPc-FA is in aqueous solution containing 1% DMSO, only a small part of monomers left in the solution (**Figure 2C**). However when some proteins are added in the solution the monomer component could increase (**Figure 2C**). If the 25% monomer component of the whole ZnaPc-FA molecules exists in the proteins contained aqueous solution such as the cytoplasm of cells, the TPE of ZnaPc-FA by a NIR fs laser is still hopeful to be effective for PDT detection and therapy.

When KB cells were incubated with 10 μM ZnaPc-FA for 2 hours, the TPE images of cells were acquired with the fs laser of 780 nm. As expected, the fluorescence image of cellular ZnaPc-FA under TPE can be clearly seen (**Figure 5A**),

indicating that ZnaPc-FA is suitable for PDT detection with TPE of fs lasers. When cells have been incubated with AlPcS (10 μM) for 5 hours, the fluorescence image is very weak (**Figure 5B**). This is because of two reasons. Firstly the cell uptake to AlPcS is comparatively low, and secondly the TPACS of AlPcS is also low.

$^1O_2$ production of ZnaPc-FA under TPE of NIR fs laser

Singlet oxygen ($^1O_2$) is believed to be the main reactive species in PDT.[27] When PS molecules are excited, they may emit fluorescence or take an inter-system cross transferring energy to surrounding oxygen molecules to produce $^1O_2$. The above TPE induced fluorescence experiments have shown that the singlet excited state of ZnaPc-FA can be reached by the TPE, so that the $^1O_2$ production of ZnaPc-FA was expected. The $^1O_2$ probe DBPF was used to detect the $^1O_2$ by adding DBPF (36 μM) in ZnaPc-FA DMSO solution during the NIR fs laser irradiation.[28] The 600 mW unfocused fs laser beam (780 nm) was used to irradiate this solution sample with the light spot of 2.6 mm in diameter and thus the irradiation power density was 110 mW/mm^2. During the irradiation, the solution was stirred by Magnetic stirrer to allow the most ZnaPc-FA can be irradiated. Then the DBPF fluorescence intensity in the irradiated sample was measured and compared with that of the sample before the irradiation. The degradation rate of DBPF was often used to evaluate the $^1O_2$ production produced in the system.[29] As shown in **Figure 6**, with the increased irradiation time the fluorescence of DBPF was monotonously decreased indicating that the $^1O_2$ was produced in this ZnaPc-FA system under the irradiation of 780 nm fs laser. Moreover, the DBPF degradation rate in ZnaPc-FA solution was ten-fold faster

than that in AlPcS solution (**Figure 6D**), demonstrating that much more $^1O_2$ was produced in ZnaPc-FA solution under TPE of 780 nm. This can be understood as the TPACS of ZnaPc-FA is much higher than that of AlPcS. Furthermore, the $^1O_2$ production of ZnaPc-FA in water solution containing 1% DMSO and 10% FBS was also comparatively measured. As shown in Figure 6C and 6D, the DBPF degradation rate in this condition is about 3-fold faster than that in AlPcS solution, but slower than that in pure DMSO solution of ZnaPc-FA. The monomer percentage of ZnaPc-FA in water solution containing 1% DMSO and 10% FBS is about 25% (Figure 2C) which is lower than that in pure DMSO solution, thus the relatively low $^1O_2$ production is understandable. However the 25% monomers of ZnaPc-FA have produced 3-fold $^1O_2$ production relative to that in AlPcS solution under TPE of a near-infrared fs laser demonstrating an encouraging picture for PDT improvements with ZnaPc-FA.

In vitro PDT killing effects of ZnaPc-FA under OPE and TPE

The MTT assay was used to measure the PDT damaging effect on cells.[30] Before the in vitro PDT experiments, every possible damaging factor was tested. The damaging effects of fs irradiation alone (without PSs) and 1% DMSO in aqueous solution on cells were found to be neglectable. When cells have been incubated with ZnaPc-FA (10 μM) for 2 h or AlPcS (20 μM) for 5 h but without light irradiations, the dark toxicity of these two PSs was also very low (**Figure 7A**). When PS incubated cells were irradiated by the common OPE of red light beam (630-730 nm) with the irradiation power density of 20 mW/cm^2, the PDT damaging was clearly shown in **Figure 7A** and the damaging extent was proportional to the irradiation dose

demonstrating a typical light dose dependent PDT model. Herein, the long incubation time (5 h) of AlPcS was to allow more AlPcS entering cells because the endocytosis process of AlPcS is found to be slow.[31] The ZnaPc-FA has the specific affinity to KB cells leading to a higher cellular amount, so that the PDT damaging effect of ZnaPc-FA was obviously better than that of AlPcS. When KB cells were pretreated with free FA and then followed by the incubation of ZnaPc-FA, the PDT killing efficiency was remarkably suppressed, confirming that the high ZnaPc-FA uptake rate of KB cells is due to the specific affinity of FA terminal of ZnaPc-FA to surface FRs of KB cells.

The TPE PDT in vitro effects of 780 nm fs laser on KB cells were further carried out. In practical PDT treatments, since the tissue scattering to the incident light is serious, focusing light in deep inside tissues is unrealistic and thus the unfocused laser beam is frequently used. To match the requirement of future PDT applications, the unfocused 780 nm fs laser beam was used in the experiment with the irradiation power density of 110 mW/mm^2 (600 mW in an irradiation spot of 2.6 mm). In TPE experiments, the KB cells were either incubated with 10 μM ZnaPc-FA for 2 h or 20 μM AlPcS for 5h. The **Figure 7B** shows the PDT killing effects of ZnaPc-FA and AlPcS. Obviously the TPE PDT efficiency of ZnaPc-FA is better than that of AlPcS. To evaluate the PDT efficacy, the overall PDT dose (light dose × incubation concentration of PS) to reach a certain death rate has been suggested as the critical parameter.[34] Herein the death rate of 60% (lethal dose, LD_{60}) was selected to qualitatively compare the PDT efficacies of ZnaPc-FA and AlPcS. According to the

data of LD_{60} in **Figure 7B**, for the ZnaPc-FA case the light dose was 600 mW×6min and the incubation concentration was 10μM, and for the AlPcS case the light dose and incubation concentration were 600mW×30min and 20μM, respectively. The overall PDT dose of ZnaPc-FA to reach LD_{60} is 10 times lower than that of AlPcS and therefore the TPE PDT efficacy of ZnaPc-FA is ten times higher than that of AlPcS. Although the cellular ZnaPc-FA may only partially exist in monomer form, the high cellular uptake rate and the high TPACS of the monomers make ZnaPc-FA still a promising PS for TPE PDT.

AlPcS is a mature PS, has been used in PDT studies for near three decades. With the registered name of Photosens, AlPcS has been approved for clinical use in Russia for the treatment of a range of cancers.[32] Comparing to AlPcS, ZnaPc-FA has better PDT characteristics including the specific targeting to FR-positive cancers and high efficacies in both OPE and TPE photosensitizations. Therefore, the ZnaPc-FA is a potential PS for PDT improvements.

CONCLUSION

ZnaPc-FA is newly synthesized compound of our group. Although the aggregation is still a problem of ZnaPc-FA in aqueous solution, benefiting from the interaction with proteins in physiological conditions the ZnaPc-FA can be partially in monomer form. Due to the function of FA terminal the ZnaPc-FA can specifically bind on the FR-positive KB cancer cells resulting in a high cellular uptake rate. Moreover, the ZnaPc-FA demonstrated the outstanding PDT abilities to kill cancer cells as compared to AlPcS. Particularly, the PDT efficacy of ZnaPc-FA under TPE of the NIR fs laser is

ten-times higher than that of AlPcS, exhibiting its potential in TPE PDT. These results suggest that the ZnaPc-FA is a promising candidate of new generation PS and worth investigating further.

Experimental Section

Synthesis of ZnaPc-FA. The conjugates of ZnaPc-FA were prepared by coupling the surface amino-group of ZnaPc with the γ −carboxyl terminal of FA with the help of DCC and NHS. The detailed procedures can be found in our previous report.[13] The solubility of ZnaPc in water was very poor. When the ZnaPc-FA was formed, the solubility of ZnaPc-FA in aqueous solution was remarkably improved. The absorption and fluorescence of samples in pure DMSO or aqueous solution were measured in spectrophotometers of Hitach U-3900 and Hitach FV2500.

Two-photon absorbance cross section (TPACS) measurements of ZnaPc-FA. The TPACS of ZnaPc-FA was measured by comparing the TPE induced fluorescence of ZnaPc-FA against that of Rhodamine B. A high-performance Mode-locked Ti:Sapphire laser (Spectra Physics Mai Tai eHP) with the pulse width of 100 fs and 80 MHz repetition rate was used for TPE. The wavelength of the output beam could be tuned from 750 - 850 nm. We focused the laser beam with a 350 mm focal length lens onto a cuvette containing the dye sample solution. The focal spot was about 200 μm in diameter. The induced fluorescence was directed onto the entrance slit of a spectrometer (Acton, Spectropro 2150i) through a multimode optical fiber. The fluorescence spectra were recorded on a liquid nitrogen-cooled CCD (Princeton,

Spec-10:100B LN) that was mounted on the spectrometer. The spectrally integrated TPE fluorescence intensity F is related to the various TPE parameters by the following formula [33],

$$F = K\Phi c l \delta I^2 / 2 \quad (1)$$

where δ is the two-photon absorption cross section and Φ is the fluorescence quantum yield. The c is the concentration of the sample. The δ_r of the reference Rhodamine B was taken to be 210 GM (1GM = 10^{-50} cm^4 s/photon).[25] δ of the sample can be expressed in terms of δ_r and other parameters,

$$\delta = \frac{F}{F_r} \cdot \frac{\Phi_r c_r}{\Phi c} \cdot \delta_r \quad (2)$$

In this measurement, the effect of hot band absorption should be noticed. As seen in Figure 1B, the absorption tail of ZnaPc-FA entends to the NIR region which is due to the thermally populated higher vibration states of the ground state (termed as hot band absorption (HBA)). The HBA also can emit the fluorescence though the efficiency is very low. When the NIR laser such as 780 nm fs laser with the high power density was used for excitations of ZnaPc-FA, the fluorescence would come from both TPE and HBA. According to formula (2) in determination of the TPACS of ZnaPc-FA, the F should be solely come from the TPE process and therefore the contribution of HBA on fluorescence needed to be subtracted from the measured integrated fluorescence intensity. The TPE is non-linear process which is only achieved under the excitation of high power density and the TPE induced fluorescence is proportional to the square of the power density, whereas the HBA is a linear process which has no requirement for the laser power and HBA induced fluorescence is simply proportional to the iradiation power. Therefore in our experiments, we used the low powers (a few mW to 30 mW) of the fs laser to do the

excitation of ZnaPc-FA obtaining the linear relationship between the fluorescence intensity and the excitation power. This linear relationship can be entended to get the value of fluorescence intensity at the higher excitation power such as 200-600 mW. Then the HBA induced fluorescence at the corresponding high power of fs laser was subtracted from the measured fluorescence of ZnaPc-FA under the excitation of high power of fs laser. Finally the TPE induced fluorescence was obtained and used to determin the TPACS of ZnaPc-FA.

Singlet oxygen ($^1O_2$) generation of ZnaPc-FA under TPE. 3-Diphenylisobenzofuran (DPBF), a sensitive probe, was used to detect $^1O_2$ during the ZnaPc-FA photosensitization under TPE. Upon oxidative degradation by $^1O_2$, the fluorescent DBPF changes to non-fluorescent o-dibenzoylbenzene[28], so that the reducing rate of DPBF fluorescence in sample solution, which is proportional to the $^1O_2$ production, can be used to measure the relative yield of $^1O_2$. In the experiment the DBPF (36 μM) was added into ZnaPc-FA DMSO solution (5 μM). The 600 mW unfocused fs laser beam (780 nm) was used to irradiate this solution sample with the light spot of 2.6 mm in diameter and thus the irradiation power density was 110 mW/mm^2. The fluorescence intensities of DBPF in the sample solution were measured after the different irradiation time of the fs laser. For comparison, the $^1O_2$ production of AlPcS aqueous solution (5 μM) was also measured in parallel under same conditions.

Cell culture and PS treatments. KB cells (human nasopharyngeal epidermal carcinoma cell lines) and A549 cells (human lung epithelial carcinoma cancer cell lines) were purchased from the cell bank of Shanghai Science Academy. These cells were incubated with DMEM medium containing 10% fetal bovine serum (GIBCO

BRL), 100units penicillin per mL, 100μg streptomycin per mL, and 100μg neomycin per mL at 37 ^{0}C in an incubator. When the cell density reached 80% confluence with a normal morphology, the different PS compounds were added into cell dishes, respectively, for a desired period of incubation in the incubator. After incubation, cells were washed three times with PBS to remove unbound drugs and fresh medium was replenished. In PS incubation of cells, the incubation aqueous solutions contained 1% DMSO for ZnaPc-FA and ZnaPc but without DMSO for AlPcS. In the specific binding experiment of ZnaPc-FA to KB cells, cells were incubated with free FA (20 μM) for one hour, then the ZnaPc-FA was added for further incubation. After PS incubations, samples were all washed with PBS three times and fresh medium were added.

Fluorescence images under OPE and TPE. A laser scanning confocal microscope (LSCM, FV1000, Olympus, Inc) with a band-pass filter of 650 - 730 nm at the detection channel and a water immersion objective ((20 ×, N.A:1.0) was used to acquire cell images. A 360 nm continuous wave was used for OPE, and a 780 nm fs laser (Coherent, Mira 900-B) was for TPE. The laser powers on cell sample were about 2 mW, measured by a power meter (Newport, Model NO. 1918-C).

Cytotoxicity assay. A new kind of MTT kit, the Cell Proliferation and Cytotoxicity Assay Kit (WST-1), was used to measure the PDT effect of the PSs on KB cells.

(1) *The PDT effects of ZnaPc-FA and AlPcS under OPE*

The KB cells with concentration of 3×10^3 cells/ml were seeded in each well of a

96 well flat bottom tissue culture plat and allowed to attach to the plat overnight. When the cells reached 80% confluence with normal morphology, the ZnaPc-FA (10μM) or AlPcS (20μM) were added into different wells and incubated for 2 hours and 5 hours, respectively. In the ZnaPc-FA incubation wells, the culture medium solutions contained 1% DMSO, whereas in the AlPcS incubation wells and other control wells the solutions were pure culture medium without DMSO. After incubation, the cells were washed with PBS three times to remove the unassociated compound and replenished with fresh medium. Then, the cell plat were irradiated by a CW light beam (630 - 730 nm) with the power density is 20 mW/cm^2. After the irradiation, the cells were incubated for 24 hours and 10 μl MTT solution (5 mg/ml) was then added into each well for incubation of another 2 hours. Finally, the optical densities (O.D) at 450 nm of each well were measured on an iEMS Analyzer (Lab-system). The cell viability in each well was determined by comparing the O.D value with that of untreated control cells in wells of the same plate. All results were presented as the mean ± SE from three independent experiments with four wells in each.

(2) The PDT effects of ZnaPc-FA and AlPcS under TPE

The KB cells were seeded in each well of a 12 well (35 mm diameter) flat bottom tissue culture plat and allowed to attach to the plat overnight. When the cells reached 80% confluence with normal morphology, 10μM ZnaPc-FA and 20μM AlPcS were added into different wells and incubated for 2 hours and 5 hours, respectively. After incubation, the cells were washed with PBS three times to remove the

unassociated compound, and replenished with fresh medium. These cells were then digested by 0.25% pancreatin for 3 minutes. The cell suspension was collected in a sterile glass tube and was irradiated by a 780 nm fs laser. During the irradiation, the cell tube was shaken to allow the most cells to be irradiated. The unfocused laser beam of 600 mW with an irradiation spot of 2.6 mm (110 mW/mm^2) was used for the irradiation. The control groups (without PS treatment) were also carried out. After irradiations, the PS treated cells and control cells were seeded in each well of a 96 well flat bottom tissue culture plat and incubated for 24 hours. Then, the MTT assay was carried out as described in last paragraph for OPE.

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Table 1. TPACS (GM) of AlPcS and ZnaPc-FA at selected wavelengths.

Wavelength/nm	780	800	820
AlPcS in water	69.9±12.4	19.4±2.6	3.7±1.2
ZnaPc-FA in DMSO	1200±124	870±70	450±10

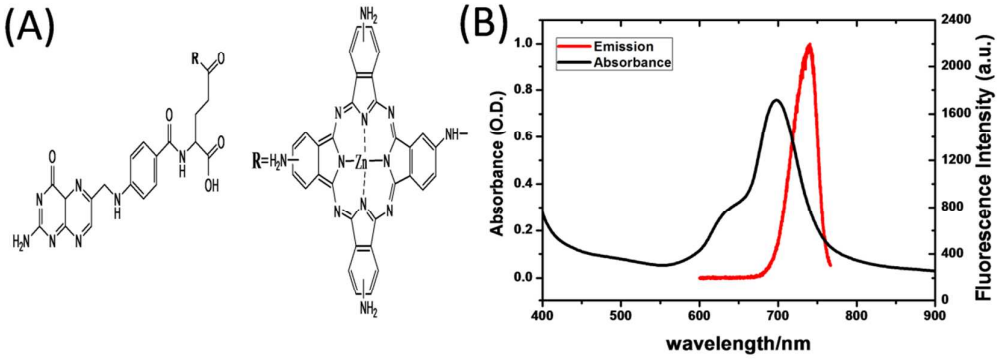

Figure 1. (A) Molecular structure of ZnaPc-FA. (B) The absorption and emission spectra of ZnaPc-FA in DMSO solution.

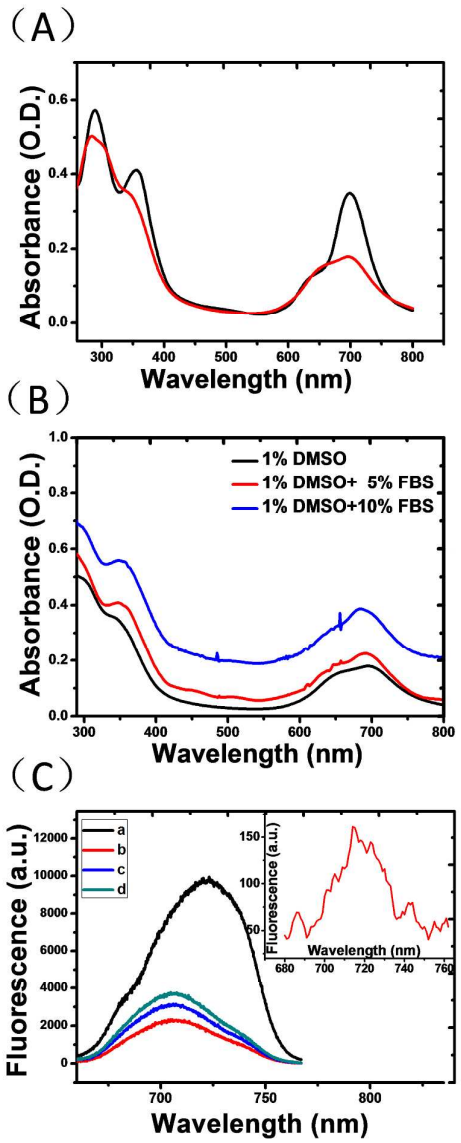

Figure 2. (A) Absorption spectra of ZnaPc-FA in pure DMSO solution (black line) and aqueous solution containing 1% DMSO (red line). (B) Absorption spectra of ZnaPc-FA in aqueous solution containing 1% DMSO with the addition of different ratio of FBS.(C) Emission spectra of ZnaPc-FA in different solution. a). Pure DMSO b). Aqueous solution containing 1% DMSO and 5% FBS. c). Aqueous solution containing 1% DMSO and 10 % FBS. d). Aqueous solution containing 1% DMSO and 15% FBS. The insert is the fluorescence spectra of ZnaPc-FA in aqueous solution containing 1% DMSO but without FBS. The concentration of ZnaPc-FA was 10μM.

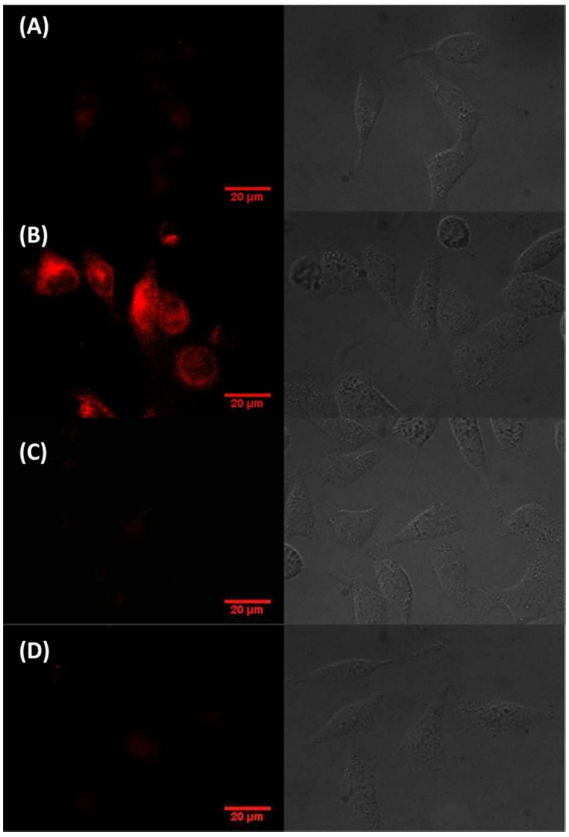

Figure 3. Fluorescence images of cellular ZnaPc-FA in KB cells and A549 cells under 360 nm OPE. Left: fluorescence images, right: DIC images.(A) KB cells have been incubated with ZnaPc (1μM) for 50min; (B) KB cells have been incubated with ZnaPc-FA (1μM) for 50min; (C) KB cells were treated with excess FA (20 μM) and then incubated with ZnaPc-FA(1μM) for 50min; (D) A549 cells have been incubated with ZnaPc-FA(1μM) for 50min.

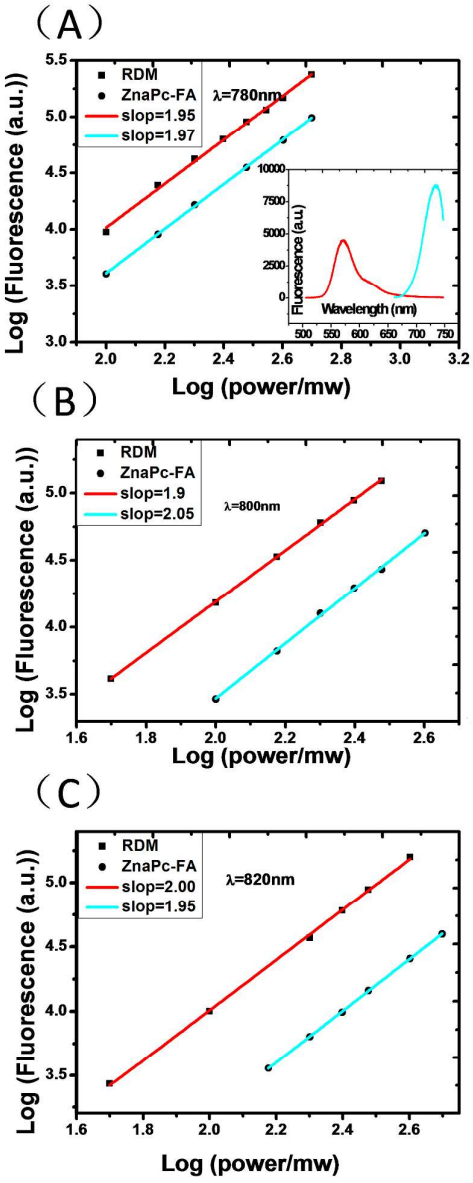

Figure 4. Logarithmic plots of the dependence of relative two-photon induced fluorescence on irradiation power density of fs laser, at different excitation wavelengths. (A) 780 nm; (B) 800 nm; (C) 820 nm. The insert in (A) shows the fluorescence spectrum of Rhodamine B (the red line) and that of ZnaPc-FA (the blue line).The concentrations for both Rhodamine B and ZnaPc-FA were 20 μM. The estimated uncertainties of the fitted slopes are ±0.1.

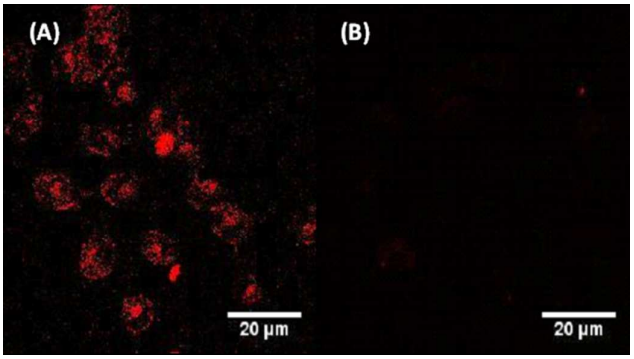

Figure 5. Fluorescence images of cellular ZnaPc-FA and AlPcS in KB cells under the TPE of a 780 nm fs laser. (A)The KB cells had been incubated with ZnaPc-FA (10μM) for 2 hours ； (B) The cells had been incubated with AlPcS (10 μM) for 5 hours.

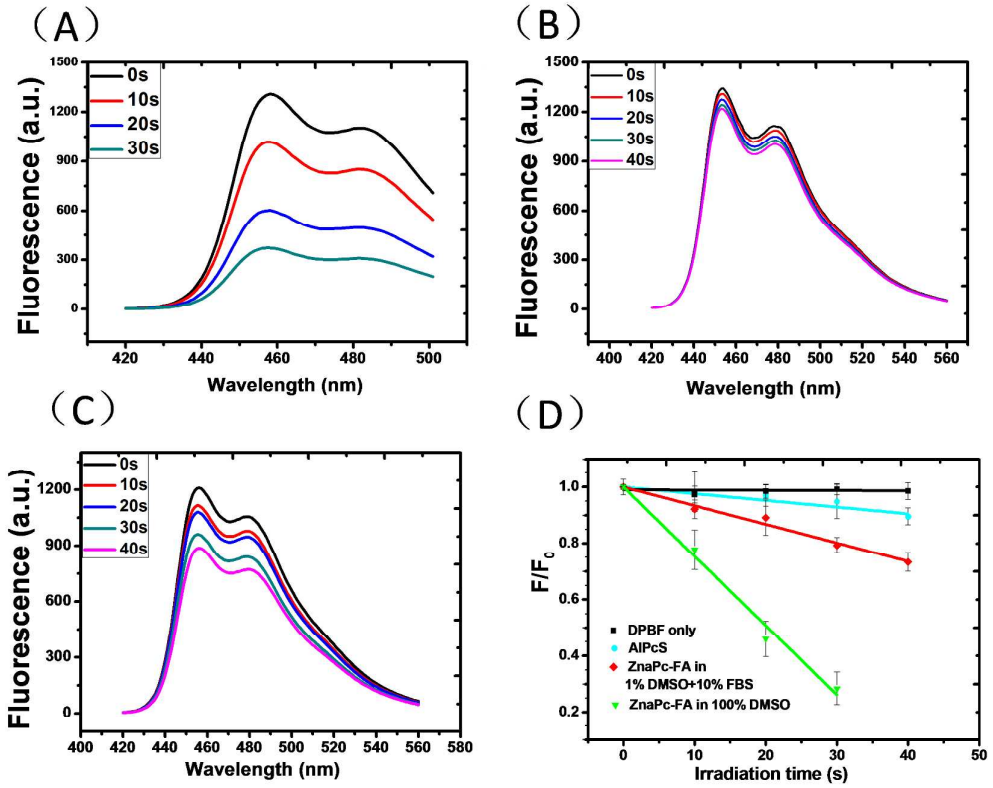

Figure 6. Fluorescence decrement of DPBF (36μM) in ZnaPc-FA (5 μM) and AlPcS (5 μM) solutions after various irradiation time of the 780nm fs laser. (A) In ZnaPc-FA DMSO solution, the DPBF fluorescence decay graph; (B) In AlPcS water solution, the DPBF fluorescence decay graph; (C) In ZnaPc-FA water solution containing 1% DMSO and 10% FBS, the DPBF fluorescence decay graph ; (D) Relative DPBF photo-degradations under the irradiation of fs laser. The black cubes represent control groups (the solution contained DPBF only), the blue spheres stand for AlPcS groups, the green triangles are for ZnaPc-FA in DMSO solution and the red curve is for ZnaPc-FA in water solution containing 1%DMSO and 10% FBS. The excitation for

DPBF fluorescence was 405 nm.

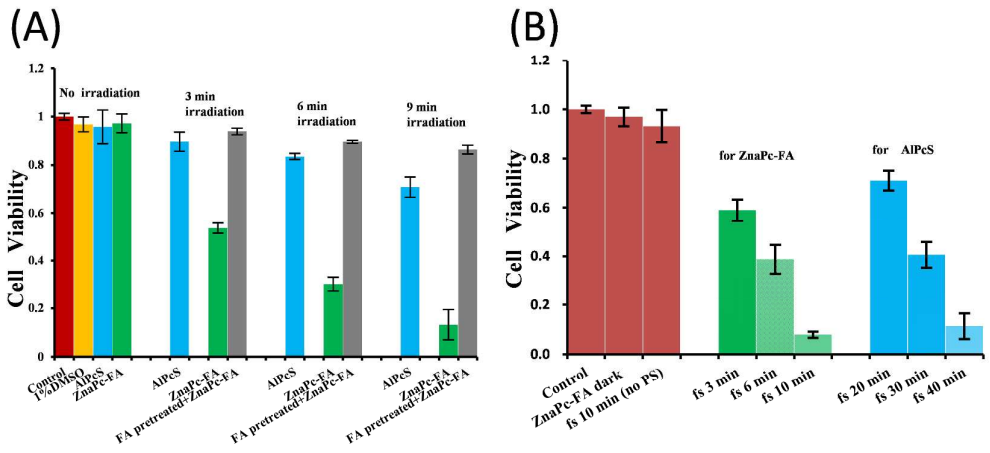

Figure.7 Viability of KB cells under different treatments. (A) The cells have been irradiated by the OPE of red light (630-730nm) (20 mW/cm^2) for different times (0 – 9 min). The control group was cell alone without PS treatment. (B) The cells have been irradiated by the TPE of the 780 nm fs laser for different times (0 – 10 min). The irradiation power density of fs laser was 110 mW/mm^2 (600 mW in an irradiation spot of 2.6 mm). In PDT groups, the cells have been incubated with either ZnaPc-FA (10μM) for 2 h or AlPcS (20μM) for 5 h. For the FA-pretreated groups, the cells have been incubated with free FA (20μM) for 2 h and then followed by the ZnaPc-FA incubation.