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Apomyoglobin is an efficient carrier for zinc phthalocyanine in photodynamic therapy of tumors

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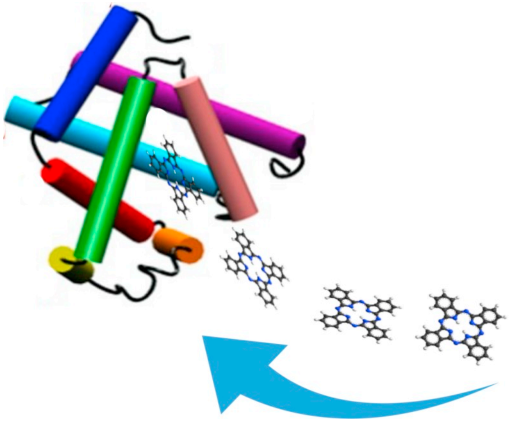
HIGHLIGHTS

- Zinc phthalocyanine binds in monomeric form to the heme pocket in apomyoglobin.
- The photosensitizer bound to apomyoglobin retains the photophysical properties normally observed in organic solvents.
- Rapid and efficient binding to cultured tumor cells indicates high bioavailability of the compound.
- The compound exerts high phototoxic effects on treated cells.

GRAPHICAL ABSTRACT



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ABSTRACT

The spectral and the photophysical properties of phthalocyanines have made these dyes attractive for applications in photodynamic therapy of cancer. One important known issue of these compounds is their tendency to aggregate in aqueous media, which decreases their fluorescence, triplet, and singlet oxygen quantum yields. We report on the use of apomyoglobin as a carrier for zinc phthalocyanine (ZnPc) to overcome solubility limitations of the dye. We show that the protein is able to bind ZnPc in monomeric form, preserving its photophysics. Confocal fluorescence imaging of PC3 and HeLa cells, treated with the complex between ZnPc and apomyoglobin, demonstrates that the photosensitizer is uptaken quickly by cells. Illumination of treated cells strongly decreases viability, as demonstrated by live/dead fluorescence assay.

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1. Introduction

Cancer photodynamic therapy (PDT) is a treatment that uses a combination of otherwise non-toxic chemicals, termed photosensitizers (PS), and visible light, to produce reactive oxygen species (ROS) capable of killing tumor cells [1–3]. Preferential accumulation of the drugs in the tumor tissue and localized illumination with visible light of suitable wavelength at optimized times allow to generate ROS, in many cases singlet oxygen ($^1O_2$), within the neoplastic tissues with good selectivity [2]. To improve preferential accumulation in cancer vs normal cells, targeted photodynamic therapy, based on different approaches, has been suggested as a promising strategy [4–7]. Efficient and precise PS loading of sensitive cellular structures is of paramount importance to achieve the desired photoinduced damages. In fact, the short lifetime of $^1O_2$, about 3 μs in water, means that the longest distance between the molecular oxidation targets and the site of $^1O_2$ generation is roughly 0.1 μm [8]. Thus, fluorescence imaging of the PS has been used to assess cellular distribution.

Phthalocyanines (Pc) have been proposed as promising PS for PDT [9] and have been the subject of several investigations aimed at improving their spectral and chemical properties [10]. These compounds have a prominent absorption band in the far red portion of the visible spectrum, ideal for an optimal light penetration in tissues, with very high molar extinction coefficient. Pcs form chelates with several metallic ions, for which photoexcitation leads to fluorescence emission, triplet formation and $^1O_2$ sensitization. In particular, z.

inc phthalocyanine (ZnPc) is characterized by an intense absorbance band at 674 nm with molar extinction coefficient 2,8 × 10^5 cm^{-1} M^{-1}, and singlet oxygen quantum yield (Φ_Δ) 0.67 [11].

A general characteristic of Pcs is their low solubility in water, which leads to aggregate formation that impairs their photophysical properties. To overcome this limitation, chemical modifications of the dyes [12,13] or the use of suitable carriers have been proposed [14–20].

The potential of proteins as carriers for Pc was explored using bovine serum albumin (BSA) as a cargo system [18,19]. BSA was shown to be fully functional in preserving fluorescence emission and singlet oxygen photosensitization of ZnPc, making it an effective PS against HeLa cells [19]. It is worth noting that some photochemical reactions with BSA amino acids lead to Trp photooxidation products [18]. Moreover, tetrasulfonated aluminium phthalocyanine was conjugated to maleylated BSA [21]. The complex was proven to be phototoxic towards macrophage-like J774 cells, whereas little activity was appreciable on the non-phagocytic EMT-6 cell line. ZnPc was also delivered conjugated to other serum proteins, in particular low density lipoprotein (LDL) [4].

We have recently used apomyoglobin (apoMb), i.e. the protein portion of myoglobin from which the heme has been removed, as a carrier for hydrophobic PSs like hypericin [22–25]. Binding of the PS to the hydrophobic pocket, normally hosting the heme, prevents PS aggregation and preserves its photophysical properties.

In this study, we explore the possibility of exploiting apoMb as a carrier for the hydrophobic drug ZnPc. Although larger in size than hypericin, we find experimentally that the dye binds to apoMb. Binding of metal phthalocyanines to apo-hemoglobin was previously demonstrated [26,27]. The complex self assembles and is characterized by fluorescence emission and triplet state formation. We show that the dye is effectively solubilized and is delivered quickly and in good yield to HeLa and PC3 cells, resulting in phototoxic effects that strongly reduce cell viability. Although devoid of targeting capability, the construct holds potential as a delivery agent in tumor PDT.

2. Materials and methods

2.1. Chemicals

Zinc-phthalocyanine (ZnPc) was from Sigma Aldrich (St. Louis, MO). Stock solutions were prepared by dissolving ZnPc in dimethyl sulfoxide (DMSO). Concentrations were determined spectrophotometrically using the molar extinction coefficient 282,000 M^{-1} cm^{-1}.

Horse heart myoglobin was from Sigma-Aldrich. We prepared apomyoglobin (apoMb) from myoglobin using standard biochemical procedures as described previously [23,25,28,29]. Solutions were prepared using phosphate buffered saline (PBS) buffer, pH = 7.4.

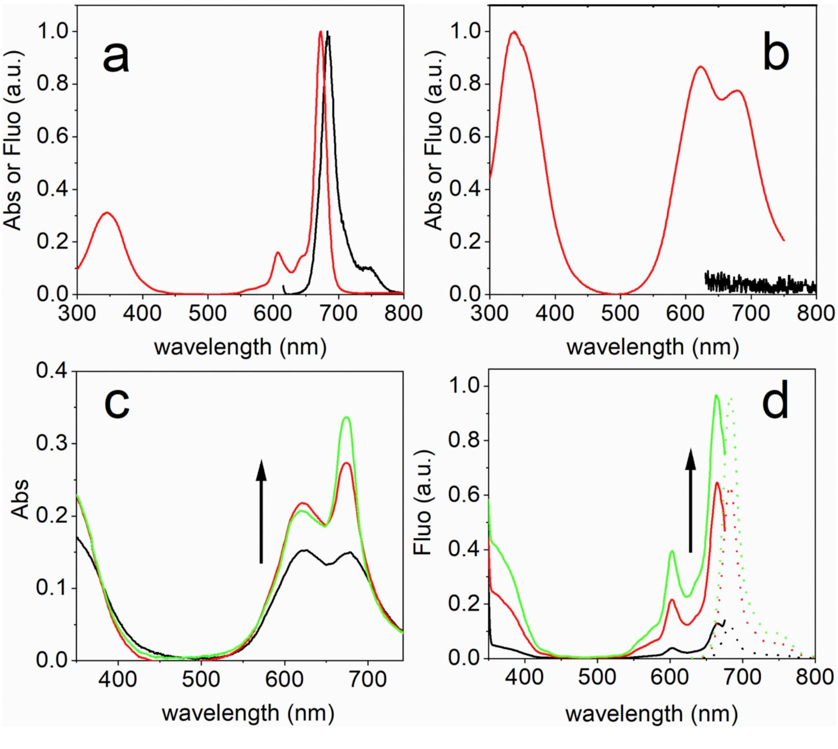

Fig. 1. Absorption (red line) and emission (black line) spectra of ZnPc in DMSO (a) and in PBS buffer (b). Absorption (c), fluorescence (d) excitation (solid lines) and emission (dotted lines) spectra of ZnPc at increasing apoMb concentrations (0.6, 4.0 and 14.3 μM). Arrows indicate increasing protein concentration. Observed changes saturate at the highest reported concentration. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

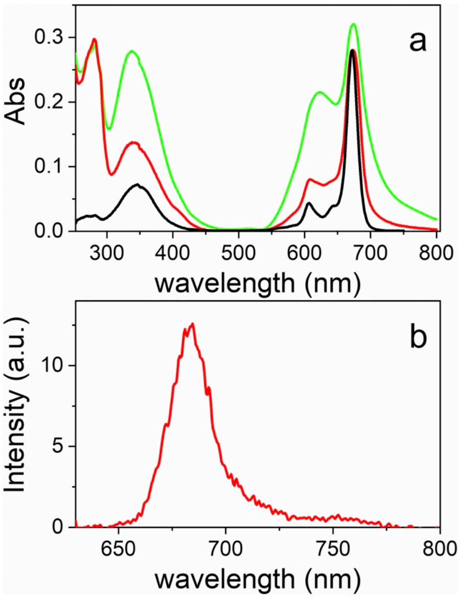

Fig. 2. Absorption (a) and fluorescence emission (b) spectra of ZnPc in DMSO (1 μM, black) ZnPc-apoMb before (green) and after (red) centrifugation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.2. Cell cultures and cell survival assays

HeLa cells were grown in DMEM supplemented with 10% Fetal Bovine Serum and 1% pen/strep and glutamine. For the viability assay, HeLa cells were seeded on Nunc (ThermoFisher) at 60–80% confluence and were used after incubation at 37 °C and 5% CO_2 for 24–48 h.

LIVE/DEAD Viability/Cytotoxicity Kit (ThermoFisher) was performed to determine the viability of cells. Calcein-AM and ethidium bromide were diluted in the medium and added to cell cultures. By intracellular esterase activity, the calcein-AM is metabolically converted by live cells in a green fluorescent product, termed calcein. Instead, Ethidium bromide is readily absorbed by dead cells, labelling DNA with a deep red emission. Cells are incubated with these reagents for 10–15 min at 37 °C and imaged by confocal spinning disk at 37 °C and 5% CO_2.

2.3. Spinning disk microscopy

We used a spinning disk confocal microscope comprising TiE inverted Microscope with Okolab incubation system, 4 laser lines (405 nm, 488 nm, 561 nm, 640 nm) and two spinning disks containing about 20,000 pinholes coupled to the same number of microlens (Yokogawa CSU-X1) necessary to focus the excitation laser light. The fluorescence light was collected by an Andor EMCCD camera Ixon3 897, characterized by high sensitivity and temporal resolution. A concentrated PBS solution of the complex ZnPc-apoMb was added to the cell buffer. Final concentration of apoMb in all experiments was kept at 30 μM to ensure full binding of ZnPc.

2.3.1. PS Uptake analysis

Fiji was used to extrapolate fluorescence profiles of 10 pixel thickness for several cells on each frame [22,30]. These profiles were analyzed using a Matlab script, where the fluorescence intensity was calculated at the plasma membrane and inside the cell as a function of time.

This analysis was applied to several cells to determine the accumulation curve. These measurements were performed using Hela and PC3 cells at the temperature of 37 °C R.H. 95% and flow 0.6 l/min.

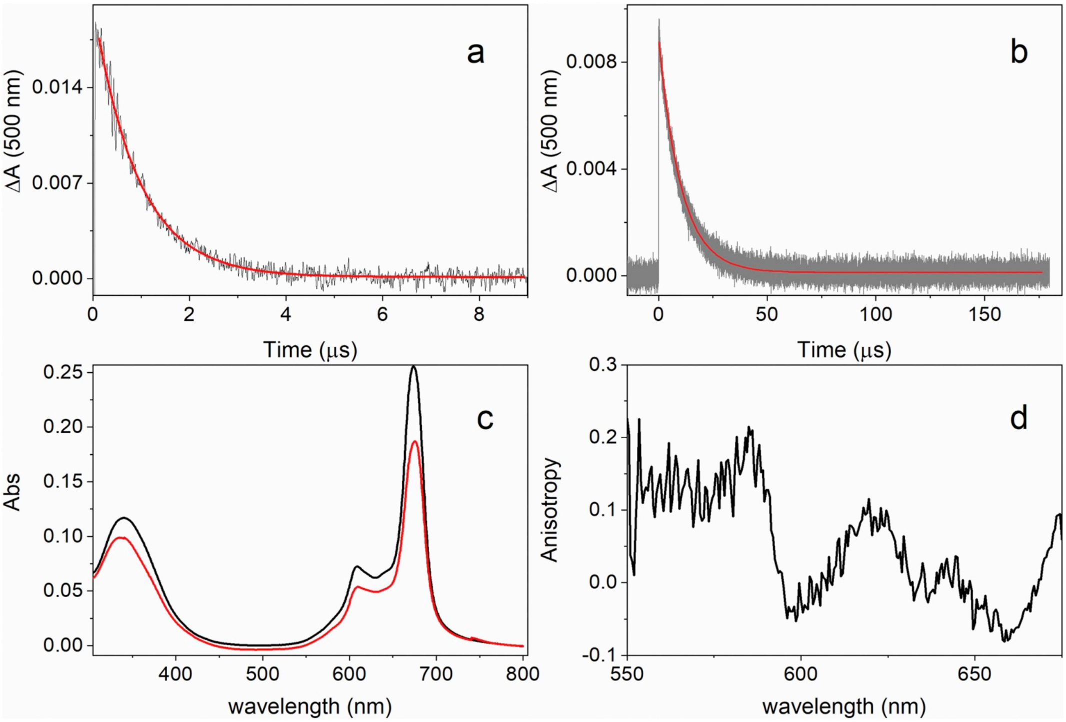

Fig. 3. Triplet state decay of ZnPc in DMSO (a, black line) and bound to apoMb (b, grey line) monitored through the triplet-triplet absorption at 500 nm. The red solid lines are the best fits to single exponential decays. Panel (c) reports the absorption spectra of ZnPc in DMSO before (black) and after (red) the flash photolysis experiment, evidencing some degree of bleaching of ZnPc. (d) Excitation fluorescence anisotropy for the complex ZnPc-apoMb. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

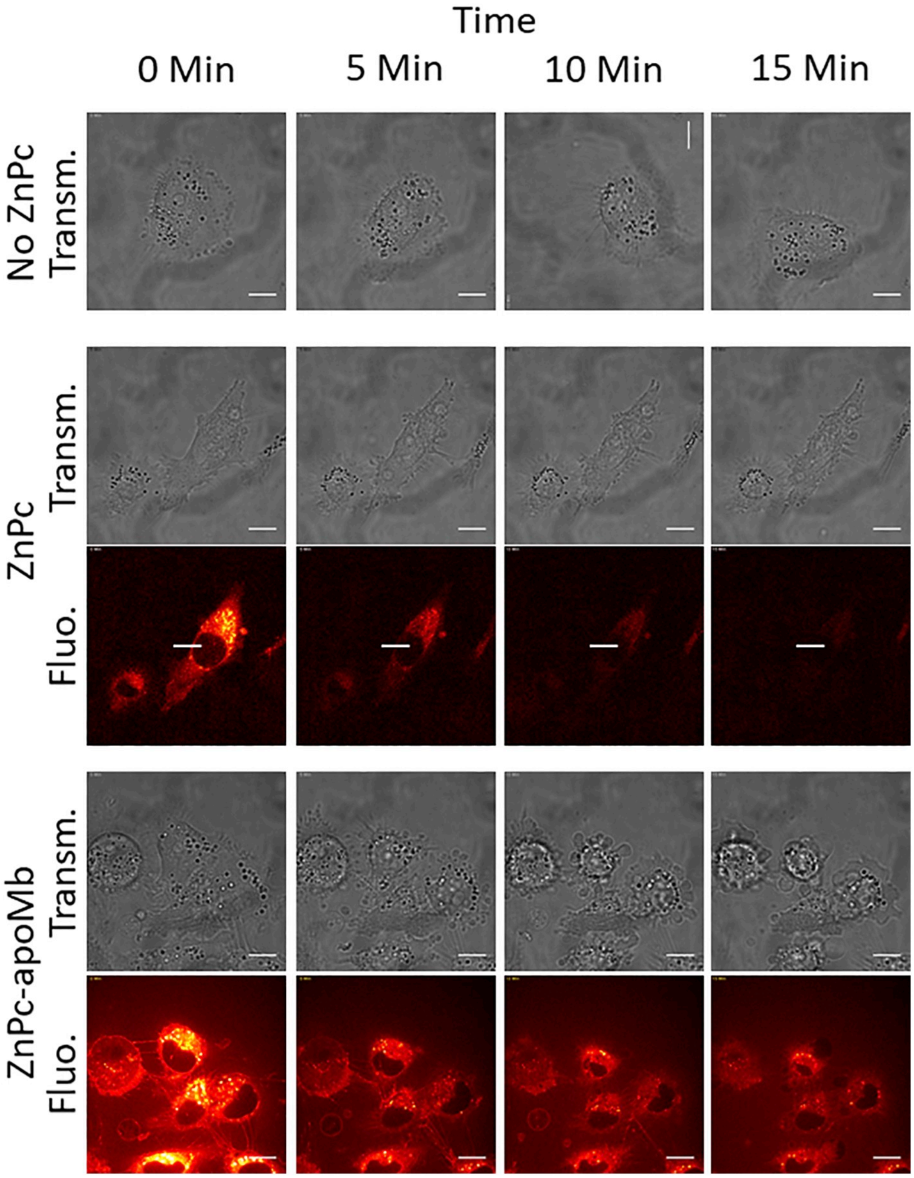

Fig. 4. First row (transmission). PC3 cells illuminated without PS. Second (transmission) and third (fluorescence) rows: effect on PC3 cells treated with ZnPc (100 nM) after 5, 10, and 15 min of illumination. Fourth (transmission) and fifth (fluorescence) rows: effect on PC3 cells treated with ZnPc-apoMb (100 nM) after 5, 10, and 15 min of illumination. ApoMb was 30 μM. Scale bar 10 μm.

2.4. *General spectroscopic instrumentation*

Absorption spectra were measured with a Jasco V-650 (Jasco Europe) spectrophotometer. Fluorescence excitation, emission and anisotropy spectra were collected with a Perkin Elmer LS50 spectrofluorometer (PerkinElmer, Waltham, MA).

2.5. *Laser flash photolysis*

Laser-flash photolysis was performed by a previously described setup [31]. Photolysis was achieved by the tunable output of an Integra (InnoLas Spitlight 300 pumping at 355 nm a GWU Versascan optical parametric oscillator). Excitation wavelength was set at 650 nm. The monitoring beam was obtained from the output of a 75 W Xe lamp (Oriel). Absorbance changes were detected through a monochromator (MS257 LOT-Oriel) with a 5-stage photomultiplier (applied Photophysics) whose output was directly fed into the 50 Ω input of a digital oscilloscope (LeCroy LT374). Triplet-triplet absorption was followed at 500 nm.

3. Result and discussion

3.1. *Spectroscopic properties of ZnPc-apoMb*

The characteristic blue colour of ZnPc in organic solvents like dimethyl sulfoxide (DMSO) is due to the intense absorption peaks in the near UV (~ 350 nm, Soret band) and the red at 674 nm (Q-band). In the solid state and within ZnPc aggregates, these bands generally split into two components due to the excitonic coupling between overlapping molecules [32].

A typical absorption spectrum of monomeric ZnPc in DMSO is shown in Fig. 1a, where it is clearly appreciable the intense absorption band peaked at 674 nm. Under these conditions, ZnPc is also fluorescent (black line in Fig. 1a), with emission peaked at 684 nm, and yield of 0.2 [33,34]. Photoexcitation of ZnPc in DMSO also leads to triplet state formation in high yield (0.5) [35].

When the ZnPc is dissolved in aqueous solutions, due to dye aggregation, the absorption spectrum changes dramatically, and the red band becomes broader and splits in two peaks (623 and 680 nm) (red

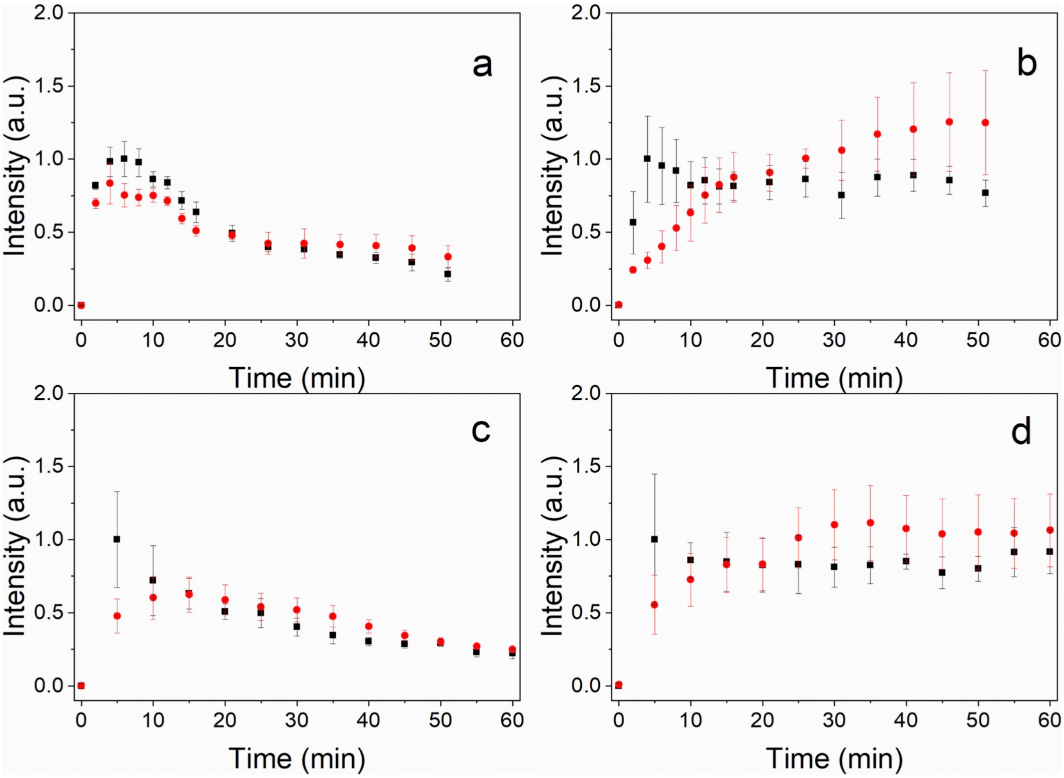

Fig. 5. Fluorescence intensity as a function of time from ZnPc (100 nM) following addition to HeLa (a) and PC3 (c) cells. Fluorescence intensity as a function of time from ZnPc (10 nM) bound to apoMb (30 μM) in HeLa (b) and PC3 (d) cells. Time 0 min is the intensity before PS injection. Black symbols, plasma membrane; red symbols, cytoplasm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

solid line in Fig. 1b). Under these conditions, ZnPc fluorescence becomes negligible (black curve in Fig. 1b). Aggregation of ZnPc leads to fast non-radiative deexcitation of the excited state and prevents triplet state formation and fluorescence emission.

Aggregation of ZnPc in aqueous buffers can be avoided if the dye is solubilized by a carrier. Following a procedure we have applied to other hydrophobic compounds [22,23,25], we have explored the possibility of using apomyoglobin (apoMb) as a carrier for ZnPc. When in the presence of apoMb, ZnPc is expected to spontaneously bind to the hydrophobic pocket normally occupied by the heme.

Fig. 1c shows the effect on the absorption spectrum of a progressively increasing concentration of apoMb in the presence of ZnPc. The band at 680 nm increases in intensity and gets sharper, becoming more similar to the spectrum observed in DMSO. The observed spectrum reflects the simultaneous presence of monomeric ZnPc molecules bound to apoMb and others aggregated in the aqueous solution.

However, for those molecules that are bound to apoMb, the protein matrix offers an apolar environment that protects their excited states, preserving fluorescence emission as can be seen in the fluorescence excitation and emission spectra in Fig. 1d. It is worth pointing out that the shape of the fluorescence excitation closely reminds that observed in DMSO, indicating that only well solubilized ZnPc molecules contribute to the observed fluorescence emission. It should be noticed that the estimate of the fluorescence quantum yield when bound to apoMb is made difficult by the presence of aggregated, non-fluorescent molecules in solution, that give a contribution to the absorption of photons at the excitation wavelength. Even at high protein concentrations, much higher than the ZnPc concentration (as in Fig. 1c), it is virtually impossible to rescue all aggregated proteins once they are formed in the buffer solution [18]. It is possible to remove those aggregates only by centrifugation (vide infra) or dialysis [18]. It is worth mentioning that ZnPc does not show any fluorescence enhancement in the presence of myoglobin (data not shown), an indication that when the heme pocket is not available, the dye is not sticking to alternative binding sites.

Since aggregated ZnPc reduces the bioavailability of the compound, due to the difficulty in recovering the molecules to a monomeric state, we have explored the possibility of removing the aggregates by centrifugation, and retrieving apoMb bound ZnPc in the supernatant.

Fig. 2a compares the absorption spectra of ZnPc bound to apoMb before and after centrifugation with that of ZnPc in DMSO. It is evident that the spectrum of the supernatant after centrifugation is close to the one observed for ZnPc in a suitable solvent like DMSO, thus confirming the presence of a (mostly) monomeric compound. Fluorescence emission is also comparable in shape (although of lower intensity) for the centrifuged ZnPc-apoMb sample and ZnPc in DMSO. An estimate of the binding constant between ZnPC and apoMb is made difficult by the high tendency to aggregate of the compound. However, when ZnPc is increasingly added in low amount to an apoMb solution, binding is quantitative, suggesting a high affinity for apoMb, as previously reported for similar systems [26,27].

Laser flash photolysis experiments were performed, to verify formation and decay of the triplet state of the compound in DMSO (Fig. 3a) and bound to apoMb in PBS buffer (Fig. 3b).

For the complex ZnPc-apoMb, we observe a longer triplet lifetime (10.3 ± 0.1 μs) in comparison with ZnPc in DMSO (0.9 ± 0.1 μs). A similar value was previously observed for hypericin bound to apoMb, for which we found a triplet lifetime of 11.1 ± 0.4 μs [25]. This suggests that the degree of shielding from molecular oxygen of ZnPc and hypericin when bound to the heme pocket is similar. The absorbance change observed for ZnPc-apoMb is approximately one half the value observed for ZnPc in DMSO, indicating a twofold reduction of the triplet yield (i.e. ~0.3). This is expected to lead to a comparable decrease in singlet oxygen yield. It is worth noting that some bleaching occurs (Fig. 3c) during the laser flash photolysis experiments on ZnPc in DMSO, where highly energetic laser pulses are used to excite the sample. A much smaller effect is observed when ZnPc is bound to

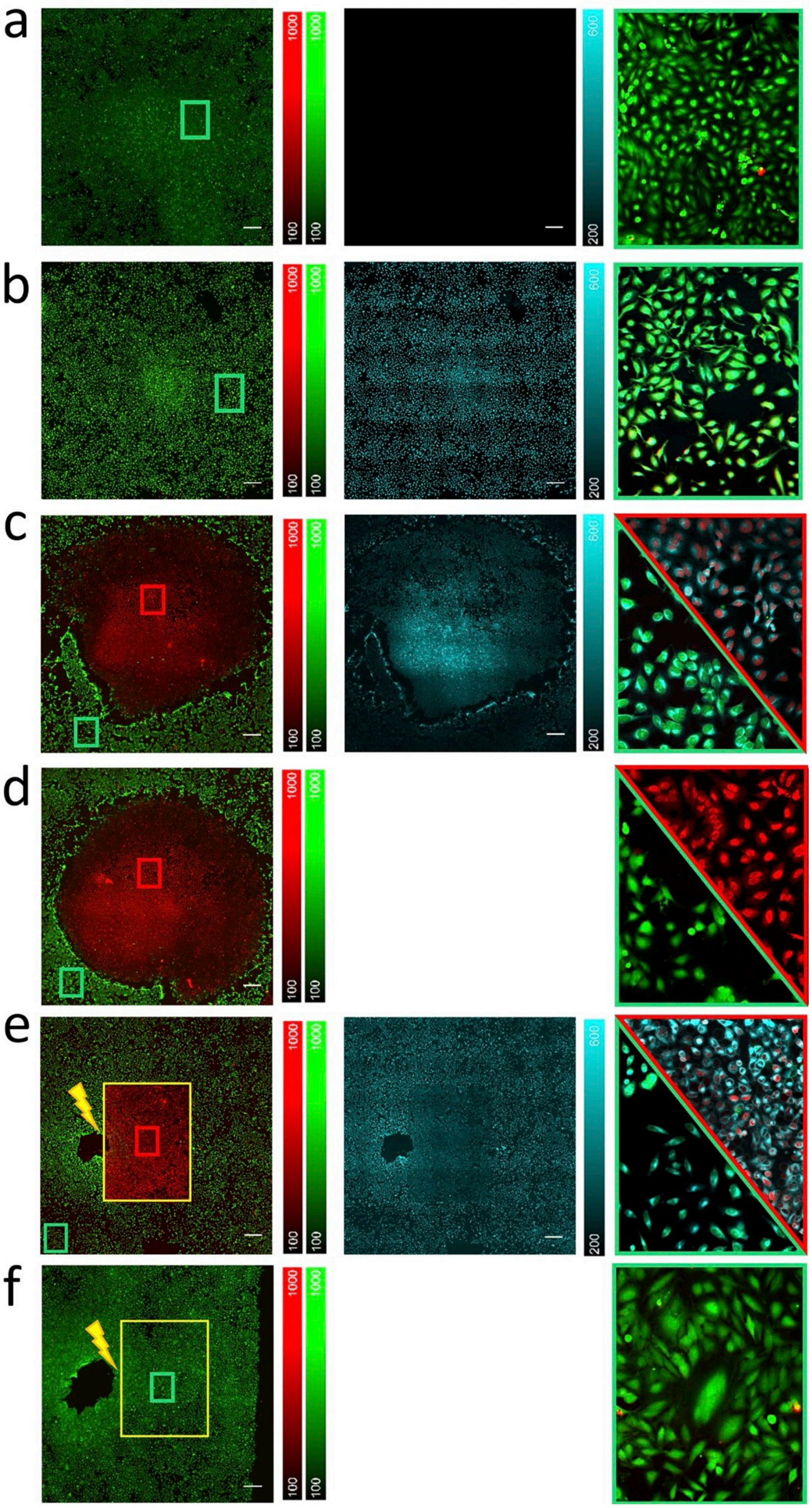

Fig. 6. Dark- and photo-toxicity assays using the lived-dead fluorescence assay. When cells are dead a red emission is observed, whereas green fluorescence emission is observed when cells are alive. ZnPc fluorescence is cyan. Scale bars are 500 μm. HeLa cells with ZnPc 500 nM in PBS (a), ZnPc + apoMb 100 nM (b), ZnPc 500 nM in DMSO (c), 5.2 μl DMSO (d). Illumination in the central region for 5 min at 647 nm with power of 2.5 mW (Irradiance 130 mW/cm^2) in the presence of ZnPc-apoMb (500 nM) (e) and without PS (f). ApoMb was 30 μM in all experiments. Light fluence for the 5 min illumination was 40 J/cm^2. Image acquisition was performed using a 4× objective 0.45NA for the large image (left and center columns) and a 20× for the inset (right column). Images are 512 × 512 pixels. The right column reports images collected on the areas with the same colored border in left column. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

apoMb, indicating a protective effect of the protein environment.

Finally, to verify binding of ZnPc to apoMb we also performed fluorescence anisotropy measurements (Fig. 3d). When ZnPc is dissolved in DMSO, its anisotropy is negligible, as expected for the fast rotational motion of the molecule. However, when the ZnPc is bound to apoMb, rotational averaging is much slower, and this results in fluorescence anisotropy different from zero (Fig. 3d).

3.2. Fluorescence imaging of ZnPc in PC3 cells

The increased solubility of ZnPc due to binding to apoMb results in enhanced availability and photosensitizing ability of the compound in comparison to pure ZnPC dissolved in DMSO. We demonstrate this concept with PC3 cells (Fig. 4). When ZnPc is administered using a concentrated DMSO solution, after one hour of incubation, uptake by PC3 cells occurs to a lower extent than when ZnPc is delivered bound to apoMb (Figs. 4, 0 min). After illumination, phototoxicity is more evident for ZnPc bound to apoMb, for which extensive morphological changes are observed (Figs. 4, 5, 10, 15 min) while for ZnPc alone it is difficult to identify clear signs of photo-oxidative damage to the cells. In this case, only bleaching of the dye is evident from the time sequence.

Image acquisition was done with a frame rate of one per minute for 19 min, laser power of (389 μW), and exposure (500 ms) with a 100 × objective, 1.4 NA. The images are 512 × 512 pixels with a pixel size of 0.249 μm/px. 8 averages were performed to minimize the noise. All measurements were done with an incubation system.

3.3. *Cellular uptake of ZnPc-apoMb*

Spinning disk confocal microscopy was used to follow fluorescence emission from ZnPc (or ZnPc bound to apoMb), beginning right after addition to the medium and for about 50 min.

In order to minimize phototoxic effects, very low ZnPc concentrations were used in these experiments, and the imaging was performed by acquiring one frame every 2 min for the first 16 min, and finally one frame every 5 min.

Fluorescence intensity at the plasma membrane and in the cytoplasm were determined following a previously established protocol [22]. Data averaged on several cells were plotted as a function of time (Fig. 5). When ZnPc is dissolved in the cell medium, the concentration of the dye rapidly increases in the cells, both for PC3 and HeLa, with a slightly higher initial concentration on the plasma membrane. Bleaching gradually reduces the fluorescence intensity in time, as already observed for the images of treated PC3 cells reported in Fig. 4. When ZnPc is delivered using apoMb as a carrier, the concentration on the plasma membrane appears to grow faster, mostly for HeLa cells, and bleaching is observed to a much lower extent. At later times, the fluorescence emission is observed rather uniformly in the cells, with no evidence of accumulation at specific sites. There is no evidence of the preferential accumulation at the plasma membrane we recently reported for hypericin [22], or indication of specific binding to cell components. Future investigations may provide additional details of the PS distributions.

Although the binding of ZnPc to apoMb is rather strong, it is unlikely that the PS is internalized as the full construct, given the lack of specific interactions of the protein scaffold of myoglobin with the plasma membrane constituents. Using apoMb labeled with fluorescein isothiocyanate, we recently demonstrated that the protein loaded with hypericin dissociates from the PS compound, and remains in the medium where cells are held, whereas hypericin is bound by the plasma membrane of HeLa and PC3 cells [22]. Thus, it seems reasonable to assume that apoMb loaded with ZnPc behaves similarly and acts as a passive carrier, enhancing the solubility of ZnPc. When in the presence of the cellular membrane, the higher affinity for the lipid phase is expected to drive ZnPc to the cellular structure, where the chemical nature of the compound determines the observed cellular distribution (Fig. 4).

3.4. *Cellular phototoxicity of ZnPc-apoMb*

Finally, three-colour large field images were acquired using live HeLa cells to assess dark- and photo-toxicity of ZnPc or ZnPc complexed with apoMb. The first two detection channels show live (green), and dead (red) cells whereas the last one is used to image ZnPc (Cyan).

Cells exposed to ZnPc (delivered in a buffer, Fig. 6a), or to ZnPc bound to apoMb (Fig. 6b), do not result in dark toxicity, (last column). The central column shows that when ZnPc is delivered in a buffer, it is nonfluorescent, whereas when ZnPc is delivered bound to apoMb, it preserves fluorescence emission. When ZnPc is delivered as a concentrated DMSO solution (Fig. 6c), dark toxicity is evident, essentially due to the presence of DMSO, as demonstrated by the control experiment where only DMSO was added to the cell medium (see Fig. 6d).

As observed above (Fig. 6b), when ZnPc bound to apoMb is delivered to the cells, its dark toxicity is negligible. But when light is shone on the sample, it quickly leads to cellular death (Fig. 6e). Control with light alone (with no PS) clearly shows no toxicity (Fig. 6f). The observed phototoxicity is remarkably high, the reduction in cell viability being observed at 500 nM and after just 5 min of illumination, corresponding to a light fluence of 40 J/cm^2.

The light-induced damage leading to cell death is confined to the illuminated area (the rectangle with a yellow border), as can be appreciated in the insets (right column, red border for the illuminated areas, green border for the non-illuminated area or for the illuminated sample in the absence of PS, panel f). The portions of the sample that were not exposed to visible light do not show any sign of reduced cell viability (cells fluoresce green, i.e. they are viable). On the contrary, portions of the sample that were treated with ZnPc-apoMb and were exposed to visible light are characterized by red fluorescent cells (i.e. they are dead).

4. Conclusions

In this work, we demonstrate that zinc phthalocyanine forms a stable complex with apomyoglobin, that preserves the photophysics of the PS in aqueous solutions. The complex can be used to deliver the PS to cultured tumor cells. Major advantages are that the uptake by cultured cells is very fast and efficient, with no evident dark toxicity. The water solubility, the high extinction coefficient in the red portion of the spectrum and the high singlet oxygen yield make this construct an efficient PS. When illuminated with visible light, a moderate fluence is sufficient to induced extensive cell death in cultures treated with nM concentrations. Chemical modifications of the protein, or fusion of the protein gene with specific peptides or proteins may allow to introduce targeting properties to the photosensitizing compound which, in its present form, is devoid of any selectivity for cell lines.

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