Study of the stabilization of zinc phthalocyanine in sol-gel TiO$_2$ for photodynamic therapy applications

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Abstract

Photodynamic therapy (PDT) has emerged as an alternative and promising noninvasive treatment for cancer. It is a two-step procedure that uses a combination of molecular oxygen, visible light, and photosensitizer (PS) agents; phthalocyanine (Pc) was supported over titanium oxide but has not yet been used for cell inactivation. Zinc phthalocyanine (ZnPc) molecules were incorporated into the porous network of titanium dioxide (TiO$_2$) using the sol-gel method. It was prepared from stock solutions of ZnPc and TiO$_2$. ZnPc-TiO$_2$ was tested with four cancer cell lines. The characterization of supported ZnPc showed that phthalocyanine is linked by the N-pyrrole to the support and is stable up to 250°C, leading to testing for PDT. The preferential localization in target organelles such as mitochondria or lysosomes could determine the cell death mechanism after PDT. The results suggest that nanoparticulated TiO$_2$ sensitized with ZnPc is an excellent candidate as sensitizer in PDT against cancer and infectious diseases.

From the Clinical Editor: Photodynamic therapy is a two-step procedure that uses a combination of molecular oxygen, visible light and photosensitizer agents as an alternative and promising non-invasive treatment for cancer. The results of this study suggest that nanoparticulated TiO$_2$ sensitized with ZnPc is an excellent photosensitizer candidate against cancer and infectious diseases.

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Key words: Titania; Phthalocyanine; Cancer; Photodynamic therapies

Photodynamic therapy (PDT) is a promising noninvasive treatment for cancer$^1$; additionally, it has been studied in a variety of nononcologic applications.$^2–4$ The process is a two-step method, in which a combination of a photosensitizer (PS) agent and UV-visible (UV-Vis) light are used in the presence of molecular oxygen to obtain a therapeutic effect. The PDT effect occurs when the PS absorbs photons (Figure 1), and the ground singlet state is an excited single state. A fraction of the excited single-state molecules is transformed via intersystem crossing into excited triplet state, forming free radicals or ions. Then, the hydrogen atom is removed and an electron is transferred to biological substrates such as membrane lipids, solvent, or molecular oxygen. These radicals interact with molecular oxygen in two steps: (1) A free electron or hydrogen atom is transferred to membrane and/or (2) the excited triplet state transfers its energy to molecular oxygen in singlet state ($^1$O$_2$) to form a highly reactive nonradical in triplet state ($^3$O$_2$). Both processes can be...
occurring simultaneously, and the ratio between them depends on the nature of the PS as well as substrate properties.2,5-9

The phthalocyanines (Pc) are a type of PS; these molecules have been studied for PDT applications,10 because they are efficiently accumulated into target cells and nontoxic for healthy cells. In addition, Pc exhibit excellent photochemical properties characterized by high singlet-oxygen quantum yields and strong absorption of far-red region wavelengths (600–850 nm).11 Zinc phthalocyanines (ZnPc) have proved to be highly promising as PSs because of their intense absorption in the red region of the visible spectrum. High triplet-state lifetimes and quantum yields are required for efficient sensitization, and these criteria may be fulfilled by the incorporation of diamagnetic metal. These kinds of molecules are lipophilic and selectively cytotoxic for tumor targets.5-7,11 This tendency suggested the study of ZnPc encapsulated in nanostructured titania to increase their photochemical properties and enhance their performance in killing cancer cells and pathogen microorganisms.3,8,9,12,13 The study of the photochemical and photophysical properties of such complexes is still very limited. In vitro studies have demonstrated the phototoxicity of ZnPc or aluminum phthalocyanine in Leishmania parasites, responsible for millions of deaths in tropical countries, especially in poor areas of the world.13-15

The use of nanoparticles as PS carriers is a very promising approach, because these nanomaterials can satisfy all the requirements for an ideal PDT agent. Most prominent among the features of successful agents is their photosensitizing ability, wherein targeted mitochondria absorb visible light; however, this feature often has been found serendipitously or empirically. In the present work nanostructured titania with ZnPc was prepared. Titanium dioxide (TiO2), is a wide–band gap (Eg = 3.2 eV) semiconductor photoactive agent with strong oxidizing power, chemical inertness, and nontoxicity.16 UV-Vis light–irradiated titania generates an electron hole pair on the surface17; pure TiO2 is photoactive against microorganisms and cancer cells when exposed to UV light, and it photocatalyzes a number of functional changes in cells.18 However, its use as a PS for PDT is restricted, because the UV wavelengths (320–400 nm) that excite TiO2 are harmful to the human body. Recently, sensitization of wide–band gap semiconductors with organic and inorganic dyes have been demonstrated.19 In this system, a PS adsorbed at the support surface is excited by visible light, and an intercomponent electron transfer is realized in the couple molecular semiconductor–semiconductor oxide, extending the useful wavelength of TiO2 from the UV to the visible region. The aim of this work is to investigate the possible synergy of zinc.
phthalocyanines supported on nanostructured titanium oxide and probe their “in vitro” photoactivity using visible light, on cancer cells and Leishmania parasites.

Methods

Synthesis of nanoparticles

The TiO₂ nanoparticles were synthesized by the sol-gel method, using two different conditions.

1. Acetic acid medium (TiO₂-ZnPc-Ace). ZnPc (Aldrich Chemical Co., Milwaukee, Wisconsin) (4.2 g, 12.36 mmol) was dissolved in acetic acid (50 mL), and then, titanium n-butoxide (85 mL) (Sigma-Aldrich, St Louis, Missouri) solution was added dropwise (4 hours). The solution was maintained under stirring until the gel was formed. Finally, the excess of water was removed at reduced pressure and the gel dried at 30°C for 8 days.

2. A similar process was used to prepare the oxalic acid medium (TiO₂-ZnPc-Oxa). The PS was prepared in the dark to avoid photobleaching.

Fourier transform infrared (FT-IR) technique: infrared spectroscopy

TiO₂-ZnPc nanoparticles were analyzed by FT-IR spectroscopy using Nicolet-710 equipment (Madison, Wisconsin). Powder samples were mixed with KBr (5 wt%) (Reasol, México, México) and pressed into thin disks mounted on a Pyrex cell with CsI windows coupled to a vacuum line. The samples were heated at 400°C under an air flux.

UV-Vis absorption spectra

The absorption spectra were obtained in the 200- to 800-nm range using a Varian (Palo Alto, California); Cary-III spectrophotometer, equipped with an integrator sphere for diffuse reflectance studies. MgO (100%) was used as reflectance reference.

Figure 2. UV-Vis spectra of TiO₂ and TiO₂-ZnPc.

Thermogravimetric analysis (TGA)–differential scanning calorimetry (DSC) technique

TGA and DSC curves were made with a Perkin-Elmer (Waltham, Massachusetts) TG-7 apparatus using a ramp from 20°C up to 800°C at 10° per minute under air flux.

Raman spectroscopy

Measurements were carried out at room temperature on a computerized Spex 1043 double monochromator (Edison, New Jersey), with the 514.5-nm line of the argon laser (lexel Laser) at a power level of 40 mW. Raman spectra were taken directly from sample powders in backscattering geometry.

Stock solutions of ZnPc, TiO₂, ZnPc-TiO₂, and the “in vitro” culture were prepared on dimethylformamide (0.1%, vol/vol).
Dimethylformamide was not toxic for the cells at the used dilution. The concentration of ZnPc on ZnPc-TiO2 was calculated considering the final weight relation of ZnPc on TiO2-ZnPc. The average particle size of each sample was measured by dynamic light scattering using a Nicomp 380/ZLS analyzer (Port Richey, Florida).

**Mammalian cells lines and parasites**

Cells and parasites were obtained from the African green monkey epithelial cells (Vero cells; American Type Culture Collection [ATCC], Manassas, Virginia); human hepatocellular liver carcinoma cells (HepG2; ATCC), human acute monocytic leukemia cell line THP-1 (ATCC), and a primary culture of human-derived fibroblasts (HDFs) were used (Molecular Probes, Eugene, Oregon). The cells were maintained by continuous culture using RPMI 1640 medium supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (Gibco, Grand Island, New York) (hFCS) at 37°C, in a humidified atmosphere of 5% CO2–95% air mixture. Adherent cell were detached with 0.25% trypsin-EDTA treatment (Gibco). The THP-1 cells were transformed to adherent cell phenotype by treatment with propylene glycol monomethyl ether acetate (10 ng/mL) for 72 hours.

**Phototoxic assays on mammalian cells**

Cell lines were prepared using mammalian cells at 2 × 10⁶ cells/mL, which were incubated with increased concentrations of TiO₂, ZnPc-TiO₂, and ZnPc in a threefold dilution series (0.05 to 50 μM) at 5% CO₂–95% air mixture and 37°C for 24 hours. After the incubation, cells were washed with fresh culture medium and exposed to red-light doses of 2.5 J/cm² using a nonionic laser light system (BFW; Edmund Industrial Optics, Barrington, New Jersey) at 670 nm (abbreviated as LS₆₇₀nm) or a photoreactor system equipped with four lamps (50 W, 120 V), 0.33 mV power, and red filters (Edmund Industrial Optics) with a spectral range of 597 to 752 nm (abbreviated as PRS₅₉₇₋₇₅₂nm). Control cells were maintained in the dark. The cell viability was calculated using the colorimetric MTT reduction assay. Twenty-four hours after irradiation, 20 μL of MTT (5 mg/mL) were added into the cells for 4 hours, and blue formazan crystals were dissolved with dimethyl sulfoxide. The optical density (OD) was measured using a microplate reader (Sensident Scan Merck, Darmstadt, Germany) at a wavelength of 580 nm. The percentage of cytotoxicity was calculated using the following equation: Cytotoxicity (%) = 1 – (OD treatment group/OD control group) × 100. The compound activity was expressed in 50% and 90% lethal concentration LC₅₀ and LC₉₀ values calculated by sigmoidal regression analysis (Mxlfit; ID Business Solution, Guildford, United Kingdom). A specific phototoxic index (PI) was calculated by dividing LC₅₀ from nonirradiated cells/LC₅₀ for irradiated cells. Values of compound activity from nonirradiated cells was referred as cell toxicity, whereas those from irradiated cells were referred as cell phototoxicity. The PI indicates how many times the compound was specifically active in the presence of light, so PI = 1 or >1 indicates no specific phototoxic activity.
Phototoxic assays on Leishmania

The performance was carried out with incubated parasites and the reference drug or the compounds by 24 hours and illuminated at light intensities of 2.5 J/cm² (ref. 14). Twenty-four hours after the treatment, inhibition of promastigotes growth was microscopically determined by counting parasite numbers in a hemocytometer. Inhibition of parasite growth was determined by comparison to untreated controls. The phototoxic effect was demonstrated by comparing the activity of the compounds with and without illumination. Each experiment was done in triplicate. Inhibitory concentration (IC₅₀) and IC₉₀ values were calculated by sigmoidal regression analysis. A PI was calculated as described above.

Cellular localization

Mammalian cells were seeded into 12-well fluorescence glass slides and incubated with 15 μM ZnPc and 100 μM ZnPc-TiO₂ in the dark at 37°C, 5% CO₂ for 24 hours. Cells were washed twice with culture medium and incubated with specific cell organelle probes. Mitochondria were stained with MitoTracker Green FM (200 nM, 1 hour, 37°C), lysosomes with LysoTracker Green DND-26 (200 nM, 1 hour, 37°C), and the nucleus with Hoechst 33342 (0.1 μg/mL, 5 minutes, 37°C) (Molecular Probes, Eugene, Oregon). After washing twice with phosphate-buffered saline, cells were examined under a fluorescence microscope (Nikon Eclipse E4000, Tokyo, Japan) using 40× magnification. Images were recorded using a charge-coupled device color digital camera (Nikon Coolpix 5000). Two independent filters were used: UV-2A filter (Ex = 330–380, DM = 400, BA = 420) for compounds and nucleus stain, and B-2A filter (Ex = 450–490, DM = 500, BA = 515) for the other probes. Red dots represented the fluorescence images of photosensitizers, the blue fluorescence represented the DNA of the cells, and the green fluorescence patterns represented mitochondrial or lysosomal organelles. When dyes and probes localize in the same organelle, the fluorescence appears as yellow-orange.

Results

UV-Vis spectroscopy

TiO₂ and TiO₂-ZnPc samples were characterized, and the photodynamic activities of phthalocyanines and TiO₂-ZnPc in the biological tests were analyzed. UV-Vis diffuse reflectance spectra of TiO₂ and TiO₂-ZnPc samples are shown in Figure 2. A well-known electronic transition at 390 nm (band gap 3.2 eV) due to TiO₂ charge transfer was observed, indicating that at this energy TiO₂ generates electron-hole pair and hydroxyl radicals. In the TiO₂-ZnPc-Ace spectrum, additional transitions were observed.

In the ZnPc spectrum two bands at 557 and 598 nm appear due to interactions between the zinc atom and the heterocyclic d-orbital of the zinc atom. In TiO₂; two bands at 256 and 400 nm, at 409 nm can be attributed to interactions between ZnPc and TiO₂ molecules, displaying TiO₂ charge transfer was observed, indicating that at this energy TiO₂ generates electron-hole pair and hydroxyl radicals. In the TiO₂-ZnPc-Ace spectrum, additional transitions were observed.

The infrared spectra

TiO₂-ZnPc samples spectra as a function of temperature are shown in Figure 3. The broad band centered at 3267 cm⁻¹ in the TiO₂-ZnPc-Ace is assigned to OH stretching vibration due to the presence of water and Ti-OH bonds. The peak at 1621 cm⁻¹ is related with symmetrical flexion vibrations of adsorbed water. This band together with the OH band disappeared when the temperature was raised to 200°C. The small peak located at 2930 cm⁻¹ is assigned to C-H asymmetrical and symmetrical stretching vibrations in the ring. Normally
metallophthalocyanines show strong peaks in the 1000–1800 cm$^{-1}$ region due to vibrations of the isoindole and pyrrole groups. The spectral pattern in this region strongly depends on the molecular structure of the complexes and of the central metal. According to Seoudi et al.\textsuperscript{22} the bands appearing at 1345, 1050, and 1027 cm$^{-1}$ are assigned to the C-N in isoindole, in plane band in pyrrole, and stretching vibration, respectively. The other two bands at 1541 and 1441 cm$^{-1}$ are attributed to the C-H in plane bending vibration. The vibrations to C-C, C-H, and C-N bonds were maintained without change up to 250°C, and decreased gradually as the temperature was raised, disappearing totally at 400°C. The spectrum measured at 400°C shows only one broadband center at 500 cm$^{-1}$ corresponding to Ti-O vibrations. The spectrum of the TiO$_2$-ZnPc-Oxa sample exhibited different bands: a sharp OH vibrations band at 3606 cm$^{-1}$; at 3311 cm$^{-1}$ N-H assigned to stretching vibrations, at 2973 cm$^{-1}$ due to C-H; at 1693 cm$^{-1}$ a strong band C = O from oxalic moiety and at 1607 cm$^{-1}$ is assigned to C-C stretching vibrations in pyrrole. At 1356 cm$^{-1}$ and 1306 cm$^{-1}$ are two stretching vibrations of isoindole ring; two peaks at 916 cm$^{-1}$ and 815 cm$^{-1}$ are assigned to C-H bending out of plane. When the organic matter was removed at 200°C, only the broadband centered at 550 cm$^{-1}$ was observed and is assigned to Ti-O vibrations.

Thermogravimetric analysis–differential scanning calorimetry

The TGA-DSC technique was used to verify the thermal stability of ZnPc in sol-gel nanostructured titania. In Figure 4 and Table 1, the percentage weight loss for the TiO$_2$-ZnPc samples is presented. The initial weight loss (3.31\%) for TiO$_2$-ZnPc-Oxa occurring between 25° and 100°C is caused by water evaporation and alcohol used during the preparation step. The second weight loss (9.74\%) between 100° and 210°C corresponds to the elimination of residual alkoxide and
hydroxyl groups. Above 200°C a gradual weight loss was observed up to 400°C, representing 40.36% of total weight loss. This corresponds to the full dehydroxylation of the solids and the decomposition of organic matter, i.e., phthalocyanines and oxalates. The residual mass (44.4%) corresponds to TiO₂.

The DSC graphs of TiO₂-ZnPc-Oxa showed two endothermic peaks at 144° and 280°C attributed to desorption of water and alkoxide; and one exothermal process at 382°C attributed to oxidation of organic matter. Similar DSC curves were obtained for TiO₂-ZnPc sample, with slight changes of the temperature and weight loss values (reported in Table 1).

Raman spectroscopy

Samples (oxalic and acetic acids titanias–ZnPc system) show great differences in Raman spectra of the original compounds like Zn-Pc, TiO₂, oxalic and acetic acids. The Raman shift frequency is characteristic of materials structure; however, in supported organometallic complexes it is common to observe lattice defects, oxygen vacancies, and effects of the particle size. Raman spectrum of TiO₂-ZnPc-Oxa shows an intense and sharp signal at 1500 cm⁻¹ assigned to pyrrole stretching vibrations. Only intense signals below 1000 cm⁻¹ that can be assigned to C-H out of plane of aromatic rings. These observations could suggest that the interaction Pc-TiO₂ takes place via the nitrogen atoms. In the sample TiO₂-ZnPc-Ace is observed a noisy line without the bands of the medium range, showing bands at higher energy (3000–3500 cm⁻¹) that must be related to O-H, N-H, and C-H vibrations.

Photosensitivity of L. chagasi and L. panamensis promastigotes to TiO₂, TiO₂-ZnPc, and ZnPc

Leishmania spp. is the causative agent of leishmaniasis, one of the major problems of public health in several tropical countries. One of the earliest events after promastigotes have entered the mammalian host is their contact with plasma proteins. It has been shown that fresh normal human serum can cause the lysis of Leishmania spp.

We propose an important alternative to treat leishmaniasis. It consists of using photoactive nanostructured TiO₂ pure or doped. Treatment with TiO₂ pure or with ZnPc-TiO₂ was not phototoxic to either species of Leishmania used (Table 2). In contrast, treatment with ZnPc was photoactive in a dose-response range, and as in the mammalian model, a higher phototoxic effect was observed after using PRS₅₉₇-₇₅₂nm. As was also described previously by Escobar et al.¹⁴ a similar range of photoactivity was observed on both species of Leishmania. No photoactivity was induced by the reference drug amphotericin B (AmB) after illumination at 2.5 J/cm² using PRS₅₉₇-₇₅₂nm (Table 2).

Intracellular distribution

Upon evaluation after 24 hours’ incubation, most of the phthalocyanine fluorescence (red color) was localized in the cell
cytoplasm. No fluorescence was detected in the nucleus. The optimal concentration of ZnPc and ZnPc-TiO₂ used was 15 μM and 100 μg/mL, respectively, as was determined by dose-response experiments. No detectable autofluorescence was present in control untreated cells. Some qualitative differences in the intensity of fluorescence signals were microscopically observed between compounds and cells. Cells treated with ZnPc-TiO₂ showed signals of lower intensity than those treated with ZnPc at equivalent concentrations. This was true for all studied cells (Figure 5). No detectable fluorescence signal was observed in HDFs after incubation with ZnPc-TiO₂ (Figure 5).

Cytoxicity

Specific molecular probes were used to determine mitochondria or lysosome (green color) colocalization of ZnPc-TiO₂ and ZnPc. An orange-yellow color was observed when the fluorescence originated from the same organelle. Mitochondrial localization was observed in Vero, HepG2, and HDFs when using ZnPc, and in Vero and HepG2 with TiO₂-ZnPc (see Figure 6). ZnPc-TiO₂ localization on HDFs was not determined due its low fluorescence signal. Lysosomal localization of ZnPc was observed in HepG2 cells after treatment.

Cytotoxicity in HepG2 culture

The aim was to evaluate the intracellular distribution of TiO₂-ZnPc and free ZnPc in both, the tumors and normal derived human cells, to determine the activity of photosensitizers and their efficiency as a potential PDT treatment for cancer.

The photodynamic activities of phthalocyanines and TiO₂-ZnPc were investigated against four different cell lines, namely human macrophage THP-1 and human hepatocellular carcinoma HepG2, Vero cells, and normal HDFs. The cells were incubated with the compounds in the dark, and the migration was analyzed by fluorescence microscopy and registered in a digital camera. Tumor and normal cells were stained additionally with specific mitochondria, lysosomal, and nuclear fluorescent markers. The efficiency of diffusion of TiO₂-ZnPc and free ZnPc into two different types of cells (tumor and normal cells) and the cell localization are discussed. The preferential localization in target organelles, as mitochondria or lysosomes, could determine the cell death mechanism after PDT.

Evaluation of photoactivity of ZnPc-TiO₂ versus ZnPc

This evaluation was carried out with THP-1, HepG2, and HDFs; these cells were 17 to 23 times more photosensitive to ZnPc than to ZnPc-TiO₂, and high PIs were observed (Table 3). Vero cells were statistically (P > 0.05) more photosensitive to ZnPc-TiO₂ than ZnPc, however, those cells showed the lowest specific PI values.

Photosensitivity of mammalian cells to TiO₂, ZnPc-TiO₂, and ZnPc

The cells were evaluated with treatment of TiO₂ alone, which was not phototoxic to the cells until the higher concentration of 50 μg/mL was reached (data not shown). In contrast, ZnPc-TiO₂ or ZnPc were photoactive at a small dose (Table 3). The phototoxic effect was associated with the irradiation system used and with the type of compound or cell tested. A higher phototoxic effect was observed using PRS$_{597-752}$nm than LS$_{670}$nm. In all experiments the maximal concentration assayed was not able to kill 90% of the cells except treatment with ZnPc on Vero cells using LS$_{670}$nm. No toxicity was induced on nontreated mammalian cells after illumination using PRS$_{597-752}$nm or LS$_{670}$nm.

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<tr>
<th>Fluency J/cm²</th>
<th>µM ± SD of ZnPc</th>
<th>Cytotoxic concentration that induces 50% of cell death.</th>
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<td>ZnPc-TiO₂</td>
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<tr>
<td>0</td>
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<td>46.33</td>
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*: Cytotoxic concentration that induces 50% of cell death.
†: Phototoxic index ( = CC$_{50}$ value at 0 J/cm²/CC$_{50}$ value after irradiation).
‡: Irradiation using a biological photoreactor PRS$_{597-752}$mm.
§: Irradiation system using a laser light LS$_{670}$mm.
<table>
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Discussion

We have studied the photosensitizing effect of ZnPc, nano-TiO₂, and ZnPc-TiO₂ conjugate, against a panel of tumor and nontumor mammalian cells and on promastigotes of Leishmania parasites. As was expected, nano-TiO₂ alone under visible light irradiation was not phototoxic for the cells; in contrast, ZnPc treatment at the same condition was photoactive for all the studied cells and parasites. The composite ZnPc-TiO₂ was not phototoxic for L. chagasi or L. panamensis promastigotes; however, it was active against tumor and nontumor mammalian cells but less than the pure ZnPc PS. ZnPc-TiO₂ was internalized by the cells at a lower level than ZnPc. The localization of ZnPc-TiO₂ and ZnPc were observed in mitochondrial cytoplasm. No fluorescence signal was observed in HDFs exposed to ZnPc-TiO₂.

References