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Exploration of metallized phthalocyanines as potential alternative photosensitizers in photodynamic therapy

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Abstract

Introduction: Photodynamic therapy (PDT) relies on the interaction of light, photosensitizers, and molecular oxygen to generate reactive oxygen species capable of inducing cytotoxic effects, particularly in cancer cells. Metallophthalocyanines have emerged as promising photosensitizers due to their strong absorption in the red region, chemical stability, and tunable photophysical properties. Understanding their coordination chemistry and interactions with biological systems is essential for optimizing their therapeutic performance. This study evaluates the behavior of various metal-bound phthalocyanines and their capacity to induce DNA photo-damage, which is central to their effectiveness in PDT.

Materials and Methods: Metallophthalocyanines containing Zn, Cu, Ni, Co, Fe, and Mn were synthesized or obtained in purified form. Their photosensitizing activity was assessed using pBR322 plasmid DNA exposed to controlled light irradiation. DNA photo-damage was analyzed using standard gel electrophoresis techniques. The photochemical reactivity of each metal-phthalocyanine complex was evaluated to determine its DNA-interaction pattern and ROS-generation potential.

Results: All tested metallophthalocyanines showed varying capacities to photosensitize and damage plasmid DNA under light exposure. Complexes containing Zn and Cu exhibited the highest DNA photo-cleavage efficiency, correlating with their favorable photophysical properties. Differences in coordination chemistry among the metal centers notably influenced ROS generation and the extent of DNA strand breaks. The results highlight structural-dependent variations in photoactivity, revealing candidates with strong potential for PDT applications.

Conclusion: Metallophthalocyanines, particularly those containing Zn and Cu, demonstrate significant potential as efficient photosensitizers for photodynamic therapy. Their ability to generate reactive oxygen species and induce DNA damage suggests promising applications in targeted cancer treatment. Further investigation into their in vitro and in vivo bioactivity will support the development of more effective PDT agents.

Keywords: Photodynamic therapy, Photosensitizer, Phthalocyanines, Reactive oxygen species, Multi-epitope Peptide Vaccine, NSCLC, Cytokine genes, Immunotherapy

1. Introduction

Photodynamic therapy (PDT) relies on the interaction of light,photosensitizers, and molecular oxygen to generate reactive oxygen species capable of inducing cytotoxic effects, particularly in cancer cells. Metallophthalocyanines have emerged as promising photosensitizers due to their strong absorption in the red region, chemical stability, and tunable photophysical properties. Understanding their coordination chemistry and interactions with biological systems is essential for optimizing their therapeutic performance. This study evaluates the behavior of various metal-bound phthalocyanines and their capacity to induce DNA photo-damage, which is central to their effectiveness in PDT [1-3].

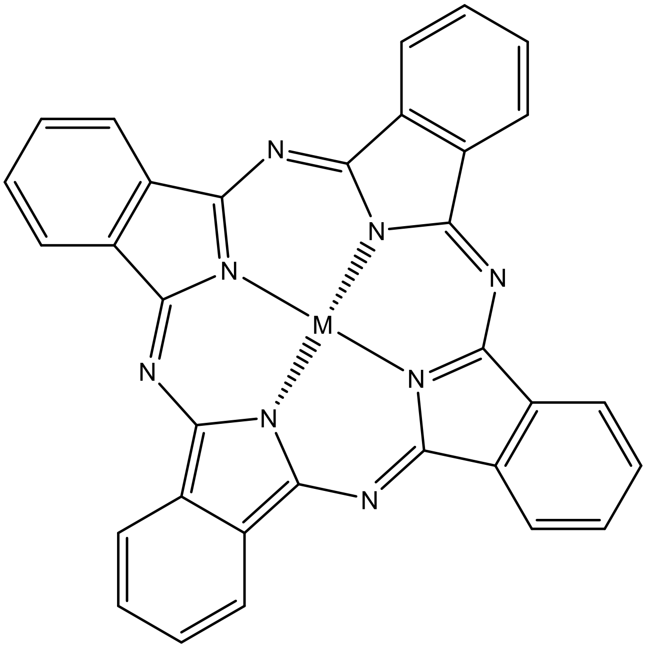

Figure 1. Phthalocyanine metal-free form H_2PC, metal ion coordinate MPC and anion. M= Fe, Co, Ni, Cu, Zn, Mn.

Phthalocyanines are organic compounds classified under the porphyrin group, characterized by their aromatic structure comprising four isoindole units. These molecules possess a unique capability to bond with a wide range of metallic and non-metallic elements at their core, imparting distinctive photochemical and photobiological properties. Notably, various metallo-porphyrins and metallo-phthalocyanines exhibit enhanced catalytic performance across a variety of chemical and photochemical processes. Examples include the photodegradation of Kraft lignin [4], the photooxidation of different unsaturated hydrocarbons [5], and the photodegradation of various organic aquatic pollutants [6]. In these processes, porphyrins and phthalocyanines are employed either dissolved in organic solutions or anchored onto various inorganic supports. This approach enhances their catalytic performance and photostability, stemming from the robust interaction between the support material and the complex [7].

The necessary conditions for their catalytic capacity are as follows: (i) the metallic ion from the macrocycle has to be redox-active, i.e., it should possess two or higher oxidation states [8], (ii) free coordination positions should be available to bind different molecules to the metallic ion [9], (iii) the coordination sphere of the metallic ion must be labile with sufficiently high exchange rates for the catalyst. Numerous researchers have discovered that the organic radical substituents and the π character, particularly the dative π effect of coordination, play a significant role in the catalytic and photocatalytic behavior of these compounds [10]. In general, phthalocyanines are compounds that are planar with highly symmetrical point groups (D4h despite the fact that their distorted form can be influenced by the bonded metal [11]. They can be synthesized through several pathways, with the most common starting materials being phthalimide, phthalic anhydride, and phthalonitrile in the presence of high-boiling solvents such as nitrobenzene. The presence of a strong base like DBU (1,8-Diazabicyclo[5.4.0]undec-7-ene) or a nitrogen source like urea along with hydrated metal salts (chlorides, acetates, etc.) is also necessary. Microwave irradiation is a more efficient, clean, and higher-yielding process for carrying out these reactions.

Phthalocyanines exhibit π-π* transitions responsible for their absorption spectra, which consist of two bands: 1) the lower-intensity Sohret band (300-400 nm) and 2) the higher-intensity Q band (650-700 nm). Furthermore, the positions of the Q bands are sensitive to the type of central metal. In general, the insertion of a metal with greater numer of electrons shifts the Q band toward the red regions due to the destabilization of the HOMO orbitals.phthalocyanines also exhibit fluorescent emission bands in the range of 650 and 700 nm. In addition, some of the phthalocyanines examined are more susceptible to charge transfer processes, which leads to a significant change in the relative position of the Q bands. For instance, manganese phthalocyanine (PcMn), characteristic Q band appears around 706-712 nm. Furthermore, luorescence quantum yields are higher for diamagnetic phthalocyanines such as PcZn and PcNi. Unlike other phthalocyanines, paramagnetic phthalocyanine (e.g., PcCu, PcCo, PcFe, and PcMn) tend to have weak fluorescent properties. The reason for this is that paramagnetic metals significantly induce intersystem crossing (ISC) processes, which simultaneously induce the deactivation of excited states and the disappearance of light emission processes. Additionally, the molecules studies on the photogeneration of singlet oxygen ($^1O_2$) showed that diamagnetic phthalocyanines tend to favor the occurrence of type II phototoxic mechanisms, while the presence of paramagnetic metals, especially Cu, Co, Fe, and Mn, reduces this possibility. However, the high probability of aggregation of the PcNi molecule may also contribute to the photogeneration of $^1O_2$. It has also been observed that certain phthalocyanines, in particular PcNi, have equal preference for activating both type I and type II mechanisms. This observation is based on the large amounts of free radicals photogenerated by this phthalocyanine during the blue tetrazolium (BTN) and chemiluminescence tests. Overall, it was concluded that diamagnetic metals with filled "d" orbitals, such as Zn, produce greater stability and improve the photosensitizing capabilities of phthalocyanine molecules, which presents a major advancement in the future development of new photosensitive compounds in our laboratory.

Phthalocyanine research is both extensive and significant, with a variety of applications ranging from medicine to technology. This includes initiation of phototoxic processes that generate reactive oxygen species (ROS) such as $^1O_2$, $\cdot$OH, and H_2O_2 through type II and type I mechanisms, respectively. Bacterial cell death can be caused by these species due to their high cytotoxicity. Based on all these considerations, we evaluated the photochemical and photobiological properties of phthalocyanines that are bound to metals of the first transition series, specifically Zn, Cu, Ni, Co, Fe, and Mn. In order to accomplish this, we replicated the microwave irradiation-assisted methods that were carried out in our lab in recent years. Unsubstituted phthalocyanine compounds have primarily been obtained through the phthalic anhydride and urea pathways. Both types of phototoxic mechanisms can be triggered by certain phthalocyanine, as demonstrated by the results of the photohemolysis tests. Among the compounds studied, the PcZn molecule exhibited the highest photohemolytic activity. The use of free radical-scavenging reagents like sodium azide (NaN_3), reduced glutathione (GSH), and superoxide dismutase (SOD) resulted in increased sensitivity to type I-mediated photoinduced damage in human erythrocytes. Several phthalocyanine molecules have phototoxic effects on DNA, particularly pBR322, a plasmid-like DNA molecule with fewer base pairs than traditional DNA. Although it is a synthetic plasmid, pBR322 has significant biomedical implications due to its genes that confer antibiotic resistance to numerous bacteria, particularly E. coli. When designing and applying photodynamic reactions, whether it is for antibacterial photodynamic therapy or for the disinfection of surfaces and biological samples, it is important to take into account the structure of the bacterial plasmid. . There are multiple ways to study DNA photosensitization. One of these methods is to perform agarose gel electrophoresis with ethidium bromide, in which each electrophoretic cartridge is loaded with a sample of bacterial plasmid- commonly pBR322 (a circular organic molecule, or a synthetic bacterial DNA)- and further monitor the respective control and irradiated samples for comparison. To accomplish this, the electrophoretic lines are read and identified as corresponding to the supercoiled (CCC), open circular (OC), and linear (L) forms of the bacterial plasmid pBR322. Evidently, once the samples are irradiated, electrophoretic mobility will depend on both mass and shape, so that supercoiled, nicked, and linear plasmids with the same mass will exhibit different migratory behavior. When utilizing agarose gel electrophoresis, phthalocyanines have shown phototoxic effects on plasmid pB322. The bacterial plasmid was found to be cleaved by phthalocyanines under irradiated conditions,and the transformation of supercoiled form

(Form I) into nicked and linear forms (Forms II and III, respectively) was observed. The significant properties of PcZn on pBR322 plasmid DNA during irradiation suggest an implicit photosensitization process. The primary cause of these effects is believed to be the oxidative reaction, mainly due to the production of large amounts of hydrogen peroxide (H_2O_2) by the aforementioned phthalocyanines. Moreover, it was revealed that irradiation of PcZn and PcCu at 650 nm, has a significant impact on plasmid pBR322 in a dose-dependent way. PcMnCl did not show significant cleavage activity on plasmid pBR322 at concentrations of 12.5 and 25 µM, although it did exhibit activity at a concentration of 50 µM at an irradiation wavelength of 750 nm. It was concluded that due to the complete transformation from the supercoiled to the nicked circular form, PcZn has the highest photo-induced cleavage capacity on plasmid DNA.. Electrophoresis was utilized to study the phototoxic properties of phthalocyanine molecules synthesized on plasmid pBR322, which is a type of bacterial DNA of great interest in biomedical research. All phthalocyanines except PcMn had significant activity on plasmid pBR322 caused by the transformation of supercoiled forms into linear and open circular forms.

This project stems from the idea of investigating how first transition series metals—specifically Zn, Cu, Ni, Co, Fe, and Mn—impact the photochemical and photobiological properties of phthalocyanines. To achieve this, we have compiled all the synthesis methods, photochemical characterization techniques, and free radical generation protocols developed in our laboratory in recent years. These procedures are intended for reproduction and further exploration in studies involving bacterial plasmid activity through electrophoresis analysis. The primary goal is to assess the effectiveness of these synthesized molecules for potential photodynamic applications. While not merely focused on Photodynamic Therapy, which involves more complex and extended clinical processes, our aim encompasses a broader range of photoinduced mechanisms that generate oxygenated free radicals. The emphasis lies in harnessing photochemical principles within biomedicine to uncover the roles of various metals in enhancing the photodynamic properties of organometallic complexes like phthalocyanines. The ultimate objective is to use these insights for the design and synthesis of advanced molecules capable of applications across diverse scientific fields.

2. Materials and Methods

All experiments were repeated at least three separate times. The graphs present mean values ± SD; $p < 0.001$ vs. control.

Metallophthalocyanines containing Zn, Cu, Ni, Co, Fe, and Mn were synthesized or obtained in purified form. Their photosensitizing activity was assessed using pBR322 plasmid DNA exposed to controlled light irradiation. DNA photo-damage was analyzed using standard gel electrophoresis techniques. The photochemical reactivity of each metal-phthalocyanine complex was evaluated to determine its DNA-interaction pattern and ROS-generation potential. Microwave-Assisted Synthesis of Phthalocyanines Bonded to Zn, Cu, Ni, Co, Fe, and Mn Metals was performed. The methods described in the following literature were used for the synthesis of phthalocyanines [12]. Using 1.48 g of phthalic anhydride, 3 g of urea, 0.012 g of ammonium molybdate, and 2.6 mmol of the metal salt. These starting products were homogenized in a mortar and pestle, then placed in a beaker and subjected to microwave irradiation for 10 minutes. For this purpose, a Mars 6 320/60 microwave reactor with a power of 2455 MHz was used. The products obtained were then washed in hot water in a beaker. After filtration, the solution was washed with various solvents, such as acetone, CH_2Cl_2, and EtOH, in a beaker. Filtration was repeated, yielding colored solids (powders). In some cases, these colored solids were treated with the acid pasting method, i.e., dissolving the washed and filtered solids in concentrated H_2SO_4, which is then recrystallized from water. In other cases, the powder obtained from the synthesis was washed with hot water, then filtered and washed several times with acetone and ethanol, without the need for acid dissolution before recrystallization.

Absorption Data: UV-VIS and Infrared Spectra

UV-VIS spectra of the synthesized phthalocyanines were taken in DMF and DMSO using a Lambda 35 spectrophotometer (Perkin Elmer). In some cases, spectra were taken in a concentrated solution (0.01 M) to observe the Sohret bands (of lower intensity, but observable in most cases at higher concentrations). In other measurements, less concentrated solutions (≈2 x 10-5 M and ≈5 x 10-6 M) were used to observe the Q band in a well-defined manner. The molar extinction coefficient (ε) was calculated using the following formula:

$\varepsilon = A/(l \times c)$ where A= Q band intensity; c= concentration (5 x 10-6 M); l = cell thickness (1 cm)

In addition, infrared spectra were taken as part of the characterization of the phthalocyanine compounds. Perkin Elmer Spectrum 100 equipment was used, employing the following conditions: # scans: 8. In KBr. Ratio: 200 mg: 0.8 mg sample. (except for PcCo, which weighed 0.42 mg); Range: 4000-4500 cm-^{1}

The infrared spectra spectrum of the phthalocyanines were carried out in a Y-IR500 Fourier transform infrared spectrometer.

Fluorescence Spectra.

To determine the fluorescence quantum yields (Φf) in DMF, an LS45 Fluorescence Spectrometer was used and corroborated with a FL6500 fluorescence spectrometer (Perkin Elmer/Corporacion científica Venezolana, C.A.) was used to obtain the fluorescence spectra of the phthalocyanines at a concentration of 7x10^{-6} M. For this purpose, the Q band values (650-670 nm) of each phthalocyanine were used as the excitation wavelength. Thus, emission bands between 650-670 nm were observed for all synthesized phthalocyanines. A comparative method was used to calculate Φf, choosing tetraphenylporphyrin (TPP) as the standard. Once the emission data for the phthalocyanines were obtained, the following formula was used to obtain the numerical value of Φf.

$$\Phi f = (\text{I ftalocianina})/(\text{I referencia}) \text{ x } \Phi f \text{ referencia}$$

Where I phthalocyanine is the fluorescent emission intensity of the band, and I reference is the fluorescent emission intensity for TPP. Φf reference is the Φf value reported in the literature for TPP in DMF (0.11).

Singlet Oxygen Quantum Yields (ΦΔ)

An indirect methodology was used to evaluate the generation of singlet oxygen from the different phthalocyanines. A standard molecule with $^1O_2$ trapping capacity, diphenyl anthracene (DPA), was used. This molecule absorbs at 373-375 nm, whereas the formation of the oxidized derivative by $^1O_2$ does not. The kinetics of disappearance were monitored by UV-Vis spectrophotometry. An LED lamp was used as the irradiation source (irradiance 3.3 mw/cm^2, wavelength 400-700 nm) and a continuous flow of oxygen was introduced into the solution composed of phthalocyanine (5 x 10^{-6} M) and DPA. The gradual decrease in absorbance was evaluated at 373 nm by successive measurements taken every 15 minutes. The following equation was used to obtain the numerical value:

$$\Phi\Delta X = (\Delta \text{Abs x})/(\Delta \text{Abs x}) \text{ x } \Phi\Delta R$$

ΦΔX= singlet oxygen quantum yield of the analyte

ΦΔR= singlet oxygen quantum yield of the reference (0.65 for TPP)

ΔAbs x= absorbance change of the specific reagent for $^1O_2$ in the presence of the analyte.

ΔAbs R= absorbance change of the specific reagent for $^1O_2$ in the presence of the reference.

Superoxide anion and hydroperoxide tests.

To determine superoxide anion ($O_2^{\cdot-}$), the NBT (Nitroblue Tetrazolium) assay was performed. In this method, the formation of $O_2^{\cdot-}$ was studied by monitoring the photosensitized reduction of nitroblue tetrazolium (NBT). This chemical process, which begins with the generation of $O_2^{\cdot-}$ through electron transfer between a photosensitizer and molecular oxygen, leads to the formation of a blue dye called diformazan, which can be detected in the spectrophotometer at a wavelength of 560 nm [13, 14]. It can be seen that the formation of a cationic intermediate (NBT+) is required for the production of diformazan. Thus, when NBT is irradiated in an air-saturated solution in the absence of a photosensitizer, no diformazan is produced. However, in the presence of a photosensitizer, the photoreduction of NBT to diformazan is evident at 560 nm.

$$1 \rightarrow 1^* \rightarrow 1 + e- \ / (O_2) \rightarrow O2^{\cdot-}$$

$$NBT + O_2^{\cdot-} \rightarrow NBT^{\cdot+} + O_2$$

$$2NBT + \rightarrow\rightarrow / (H+) \rightarrow DF$$

To perform these experiments, 50 µl of phthalocyanine solutions at a concentration of 5x10-5 M dissolved in 4 ml of a 5x10^{-5} M NBT solution were used. Irradiation was carried out in a LuzChem irradiation reactor LZC, with white light sources (visible spectrum). The samples were irradiated for a total of 80 minutes, with absorbance changes measured every 20 minutes during irradiation. Furthermore, the ability to "trap" -OH radicals was determined by luminol chemiluminescence after irradiation with a visible light source for each of the synthesized phthalocyanines. It is assumed that as the number of oxygenated radicals increases, the intensity of chemiluminescent light also increases [15].

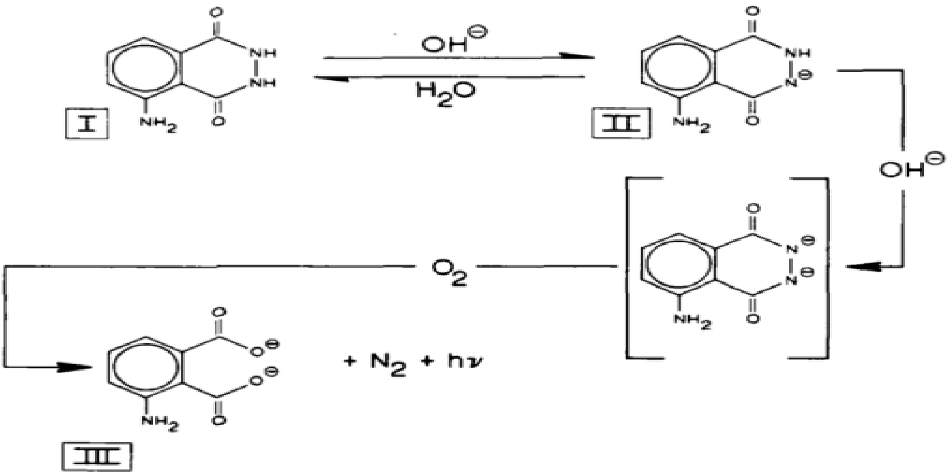

Figure 2. Chemiluminescent reaction.

For this, hydrogen peroxide (H_2O_2) (3.5 mM) is added to a phosphate-buffered saline solution (PBS, 10 mM KH_2PO_4, and 150 mM NaCl, pH 7.2) and luminol (5-amino-2,3-dihydro-1,4-phthalazinedione, Aldrich, Milwaukee, MN, USA) (250 µM, prepared in 2 M NaOH and diluted with PBS). Chemiluminescence induced by the generation of oxygenated free radicals by phthalocyanines was measured after irradiation with a visible light source in the presence of NADPH. The chemiluminescence generated at 37 °C was then continuously measured in a Luminoskan Ascent Luminometer, (ThermoLabsystems, Finland) in a 96-well ThermoLabsystems microtiter plate, using 96-well microtiter plates. In our case, 10 continuous measurements of chemiluminescent light intensity were made in order to construct decay curves and thus estimate the oxygen-free radical generation capacity of each of the phthalocyanines.

The methodology used in detecting oxygen-free radicals by chemiluminescence was repeated again, but in the presence of the enzyme superoxide dismustase (SOD). This is because NADH cannot discriminate between the types of radicals produced by the type I mechanism. Therefore, the addition of SOD allowed us to further understand the mechanism involved, as this enzyme is capable of reacting with the superoxide anion, and generating a decrease in chemiluminescent light intensity. This made it easier to determine which type of free radical may be involved in the process, depending on the type of phthalocyanine. For the experiments, 20 µl of NADH (0.1 mM) and 20 µl of a $5x10^{-5}$ M phthalocyanine solution were placed in the chemiluminescence microwells and irradiated with a visible light source in a LuzChem reactor for 10 minutes. Chemiluminescent light intensities were then measured in the Luminoskan Luminometer by manual injection of Luminol (20 µl). The SOD concentrations used were approximately 3,900 units/mg, with 15 µl of this solution added where appropriate.

3. Results

All tested metallophthalocyanines showed varying capacities to photosensitize and damage plasmid DNA under light exposure. Complexes containing Zn and Cu exhibited the highest DNA photo-cleavage efficiency, correlating with their favorable photophysical properties. Differences in coordination chemistry among the metal centers notably influenced ROS generation and the extent of DNA strand breaks. The results highlight structural-dependent variations in photoactivity, revealing candidates with strong potential for PDT applications.

Phototoxic processes and DNA damage.

The study of phototoxic processes in DNA was performed using agarose gel electrophoresis and ethidium bromide, where each electrophoretic cartridge was loaded with a bacterial plasmid sample, commonly pBR322 (a circular organic molecule, or synthetic bacterial DNA). The respective control and irradiated samples were monitored for comparison. To this end, phthalocyanine solutions were prepared at different concentrations, and various amounts (between 5, 10, and 20 µl of the phthalocyanine solution) were added to a 20 µl sample [16]. The samples were then irradiated using a visible light source in a LuzChem reactor. The total irradiation time ranged from 20 to 30 minutes. Subsequently, approximately 4 µl of the irradiated samples were injected into the electrophoretic cartridges. The electrophoresis was carried out on a 1% agarose gel with 5 ml of ethidium bromide (EtBr, 0.5 $mg.L^{-1}$), applying a voltage gradient of 40-45 V.cm-1 and a current of 60 mA. The total electrophoresis run time was 1 hour for all electrophoresis experiments. It should be noted that in all cases, a space was reserved for samples of pBR322 alone (irradiated and non-irradiated), as well as for samples of pBR322 in the presence of different non-irradiated phthalocyanines. This was done to determine possible toxic effects of the "darkness" of the phthalocyanines in pBR322, and also to rule out the possibility of the irradiation source causing damage to the plasmid. Finally, the electrophoretic lines were read and identified using an UPLAND UV Transilluminator . Depending on the case, the bands corresponded to the supercoiled (CCC), open circular (OC), and linear (L) forms of the bacterial plasmid pBR322,. Evidently, once the samples are irradiated, electrophoretic mobility depends on both mass and shape, so that supercoiled, nicked, and linear plasmids will exhibit different migratory behavior. This migration is encouraged and enhanced by increasing the concentration of the agarose gel and applying a high electric field. Experiments with pBr322 were always performed with control samples in the dark, and the results, when compared with irradiated assays, were relatively zero.

Photoinduced hemolysis of erythrocytes.

The photohemolysis experiments and those used to determine superoxide ion and singlet oxygen, were always monitored with controls in the dark and under an argon atmosphere. To study the phototoxic mechanisms of synthesized phthalocyanines in greater depth, photoinduced hemolysis (photohemolysis) experiments were performed using human erythrocytes. To this end, fresh human blood was drawn, and the plasma and blood erythrocytes were subsequently separated. Afterwards, solutions of human erythrocytes were prepared in PBS buffer (sodium chloride NaCl: 137 mM; sodium phosphate Na_2HPO_4: 2.7 mM; potassium phosphate K_2HPO_4: 2.7 mM; pH = 7.4), and 50 µL of the phthalocyanine and 15 µL of specific additives (NaN_3, GSH, and SOD) were added to allow a closer examination of the potential phototoxic mechanisms of phthalocyanines.

The erythrocyte solutions containing phthalocyanines were irradiated with white lamps (visible spectrum) in plastic cells at an irradiance of 3.30 mW/ cm^2 for 40 minutes in a LuzChem reactor. The decrease in absorbance was observed at 545 nm for every 10 minutes during irradiation. The irradiated samples were compared with non-irradiated samples to determine the effect of phthalocyanine-induced photodamage. These in vitro tests are very useful for the characterization of photosensitizers, as they provide information about the mechanisms involved in their photobiological properties. In this sense, it is a measure that allows determination of the effects of metals on phthalocyanines with respect to their interaction with and damage to biological cell membranes [17]. An aspect worth emphasizing in this regard is that the purpose of performing photohemolysis tests is to determine which free radicals may be involved in potential phototherapeutic applications of phthalocyanines. Interaction with erythrocyte plasma membranes is not an easy issue to correlate, especially when we focus on photodynamic applications for the elimination of bacteria. Essentially, the photodamage induced by phthalocyanines in human erythrocytes cannot be related to the damage caused to bacterial plasmids, since the bacterial membrane,comprising a very complex membrane envelope, is very different from that of human erythrocytes. This idea is based on the fact that to damagebacterial plasmids, which are critical for the survival of bacteria, it is necessary tobreak through the complex barrier that protects them from external elements [18]. Therefore, this study was limited to merely examining the mechanisms with the potential of producing the desired therapeutic effects, regardless of the type of interaction (bacterial or erythrocytic) with the membranes. Red blood cell and plasma samples were voluntarily collected fresh by Miguel León (an associate of this project) following our institute's biomedical standards. They were immediately centrifuged and washed with PBS pH 7.2 for analysis. DNA, superoxide dismutase (SOD), NBT, sodium azide NaN3, and pBr 322 were obtained from Sigma-Aldrich.

Electrophoresis technique

The electrophoresis run was carried out on a 1% agarose gel with 5 ml of ethidium bromide (EtBr, 0.5 mg.L-1), applying a voltage gradient of 40-45 V.cm-1 and a current of 60 mA. The total electrophoresis run time was 1 hour for all electrophoresis experiments. Figure 8 describes the photoinduced activity of the different metallized phthalocyanines on pBR322 analyzed by the agarose electrophoresis technique. It should be noted that in all cases, a space was reserved for samples of pBR322 alone (irradiated and non-irradiated), as well as for samples of pBR322 in the presence of the different phthalocyanines. All this was done to determinepossible toxic effects of the "darkness" of the phthalocyanines in pBR322, and also to rule out the possibility of the irradiation source causing damage to this plasmid. Finally, the electrophoretic lines were read and identified using an UPLAND UV Transilluminator . Depending on the case, the bands corresponded to the supercoiled (CCC), open circular (OC), and linear (L) forms of the bacterial plasmid pBR322, Evidently, once the samples are irradiated, the electrophoretic mobility depends on both mass and shape, so that the supercoiled, nicked and linear plasmids will exhibit a different migratory behavior. This migration is encouraged and improved by increasing the concentration of the agarose gel and applying a high electric field [19].

UV-VIS Spectra

UV-VIS spectra of phthalocyanines were taken in polar solvents, specifically DMF and DMSO, using a Lambda 35 UV/VIS spectrometer i.e.,PerkinElmer. In general, the typical phthalocyanine bands (Q and Sohret bands) were observed. The location of each of these bands varied depending on the solvent used in each case. In the following, Table 1 explains the results of the UV-VIS spectra of phthalocyanines in DMSO along with the molar absorptivity data for the Q bands and melting point values.

Further, Table 2 displays the characteristic UV-VIS bands of phthalocyanines in DMF and the molar absorptivity data for the Q bands. And fFinally, Table 3 shows the most representative bands of phthalocyanines in the IR spectrum carried out in a Y-IR500 Fourier transform infrared spectrometer.

Table 1. UV-VIS spectra of phthalocyanines in DMSO

Compound	Q Band	Sohret Band	Molar bsorptivity molar (ε)
PcZn	672 nm**	343 nm	132.000
PcCu	674 nm*	not detectable	35.569
PcNi	669 nm*	381 nm	37.926
PcCo	656 nm**	323 nm	44.978
PcFe	670 nm**	not detectable	19.444
PcMn	716 nm**	354 nm	23.336

Spectrum taken at 2x10-6 M; **Spectrum taken at 2x10-5 M

Table 2. UV-VIS spectra of phthalocyanines in DMF

Compound	Q band (nm)	Sohret band	Ɛ (L M^{-1})^{a}
PcZn	669*	341^{b}	123.255
PcCu	667**	not detectable	13.690
PcNi	666**	333	43.550

PcCo	658*	323[b]	107.240*
PcFe	667**	not detectable	15.306**
PcMn	707*	349 nm[b]	56.785*

*2 x 10^{-5} M; **2 x 10^{-6} M

Table 3. Most representative bands in the IR spectrum of phthalocyanines (data were obtained from multiple Samples).

	PcZn	**PcCu**	**PcNi**	**PcCo**
C-N (aliphatic amine)	1082, 1088	≈1000- 1100	1068, 1090	1121, 1090
C=C (aromatics)	1402-1613	≈1390, 1470, 1500	1428, 1471	1641, 1630
C=N aliphatic amine) (conjugation effect)	1715	≈1700	1699	1659
Bands N-Metal	530, 588, 663, 783, 894	≈700	915, 726, 573, 455	910, 724, 544

Fluorescence Spectra

All fluorescence spectra of phthalocyanines were taken in DMF in an LS45 Fluorescence Spectrometer and corroborated in a FL6500 fluorescence spectrometer (Perkin Elmer/Corporacion Científica Venezolana, C. A.). For this purpose, each of the Q bands of these molecules was used as the excitation wavelength. The emission bands were observed in the 650–670 nm range, and the intensities of each emission band were taken to calculate the fluorescence quantum yields Φ_F, following the comparative method. Tetraphenylporphyrin was used as a fluorescent probe for comparison. Table 4 details the fluorescence values obtained, as well as the fluorescence quantum yield calculation.

Table 4. Fluorescence of unsubstituted phthalocyanines bonded to Zn, Cu, Ni, Co, Fe, Mn in DMF (≈7x10^{-6} M)

[a]**Compound**	*** Q band**	**Longitud de excitación**	**Emission Length**	**Emission intensity**	Φ_F
PcZn	669 nm	669 nm	669 nm	128	0.098
PcCu	667 nm	667 nm	667 nm	22.23	0.017
PcNi	666 nm	666 nm	666 nm	136.67	0.10
PcCo	658 nm	658 nm	659 nm	56	0.042
PcFe	667 nm	667 nm	667 nm	22.98	0.017

PcMn	707 nm	707 nm	712	2.76	0.002
TPP**	422 nm	422 nm	426 nm	143.8	***0.110

^{a}Concentration ≈7x10^{-6} M; *Band with the highest number of ε; **Reference compound; ***Value reported in the literature.

Singlet oxygen generation assay using diphenylanthracene (DPA) as a probe.

To detect photoinduced singlet oxygen generation by phthalocyanines, an indirect method was used that utilizes a "trapping" probe for this species. In our case, diphenylanthracene (DPA) was used as the probe, as it is highly selective and specific for singlet oxygen. Tetraphenylporphyrin (TPP) was used as the compound allowing the comparative method to be performed.

Table 5. Singlet oxygen generation by phthalocyanines.

Compound	**ΦΔ**
PcZn	0.2438
PcCu	0.0499
PcNi	0.1083
PcCo	0.0672
PcFe	0.0704
PcMn	0.0477
TPP*	0.65**

* Compound used to perform the comparative method. **Value reported in the literature [13].

Superoxide anion and hydroperoxide test. The results of the photoinduced superoxide anion generation test are demonstrated in figure 3 and 4. The chemiluminescence of irradiated phthalocyanine and the results of the photoinduced hydroperoxide generation test are shown in figures 5 to 7.

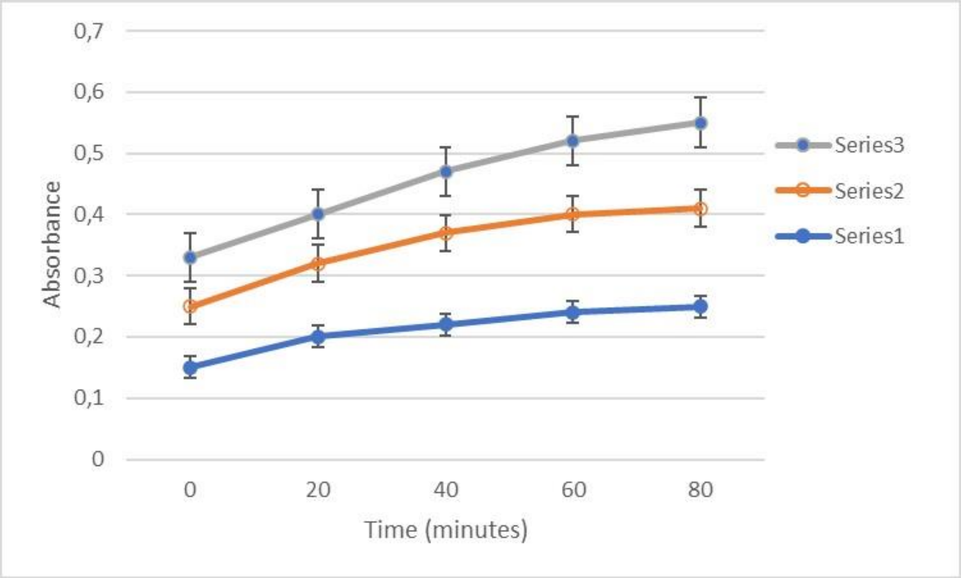

Figure 3. Superoxide anion generation as a function of time by phthalocyanines using the NBT test. The graphed curves were taken from three experiments performed for each compound; $p < 0.05$ vs. control. Serie 1 is PcZn, Serie 2 is PcCo and serie 3 Pc Ni; No generation of superoxide anion species was detected in the presence of Cu, Mn, Fe phthalocyanines.

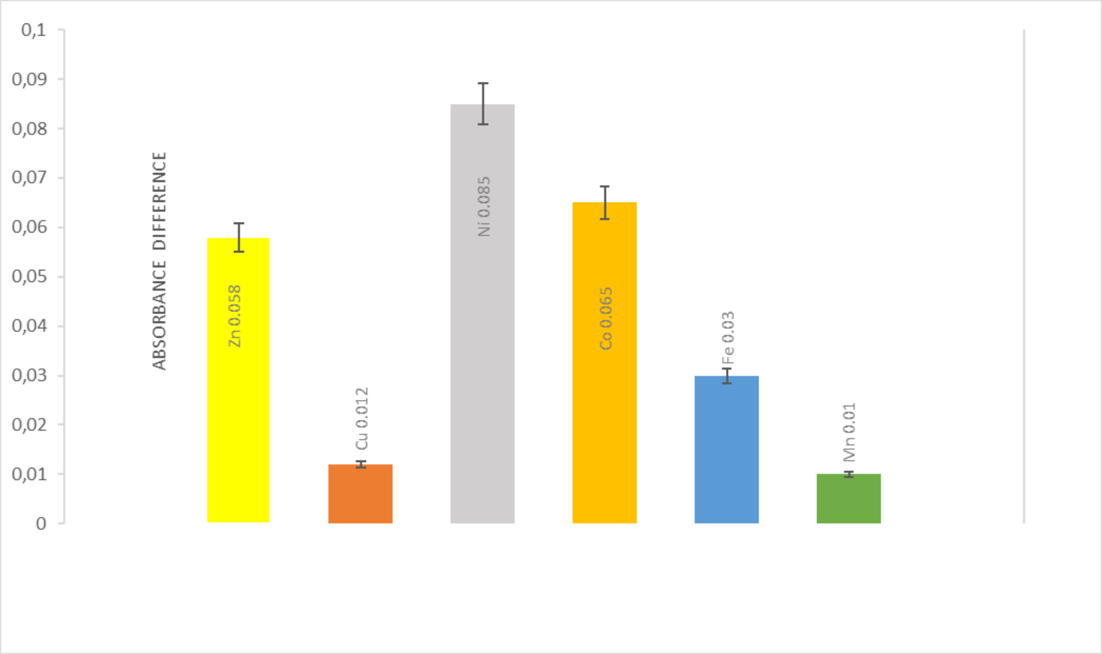

Figure 4. Superoxide anion generation by phthalocyanines using the NBT test. Representative blot images are presented; $p < 0.001$ vs. control.

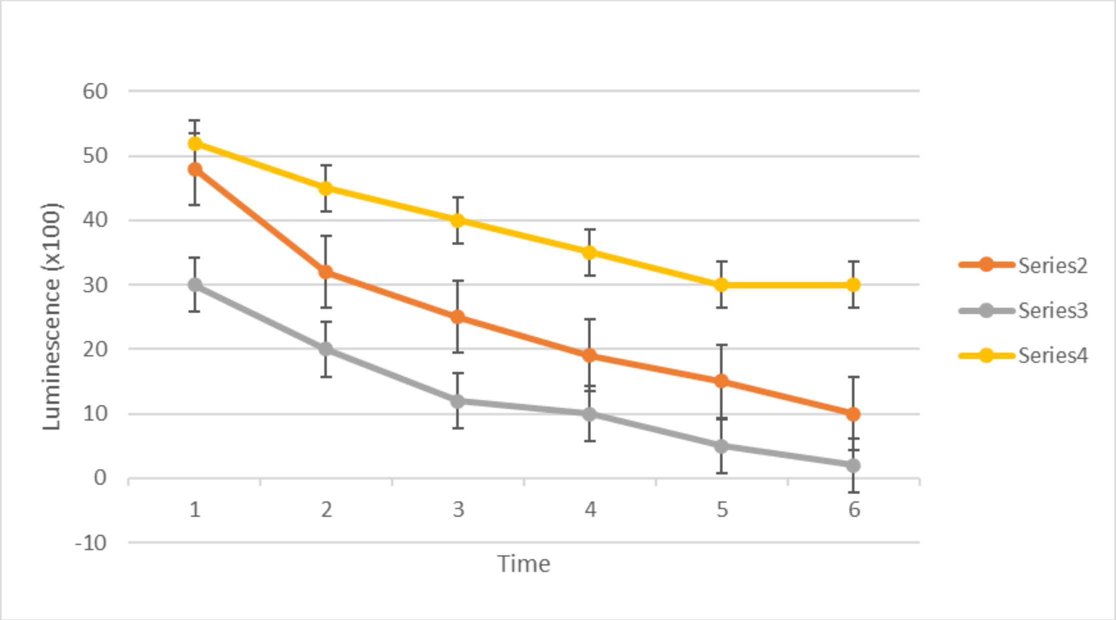

Figure 5. Chemiluminescence of irradiated phthalocyanines in DMF, without the presence of superoxide dismutase (SOD). $p < 0.05$ vs. control. Serie 4 is H_2O_2 + luminol (control), Serie 2 is PcNi and serie 3 PcCo; No generation of Luminescence was detected in the presence of Cu, Mn, Fe, Cu phthalocyanines.

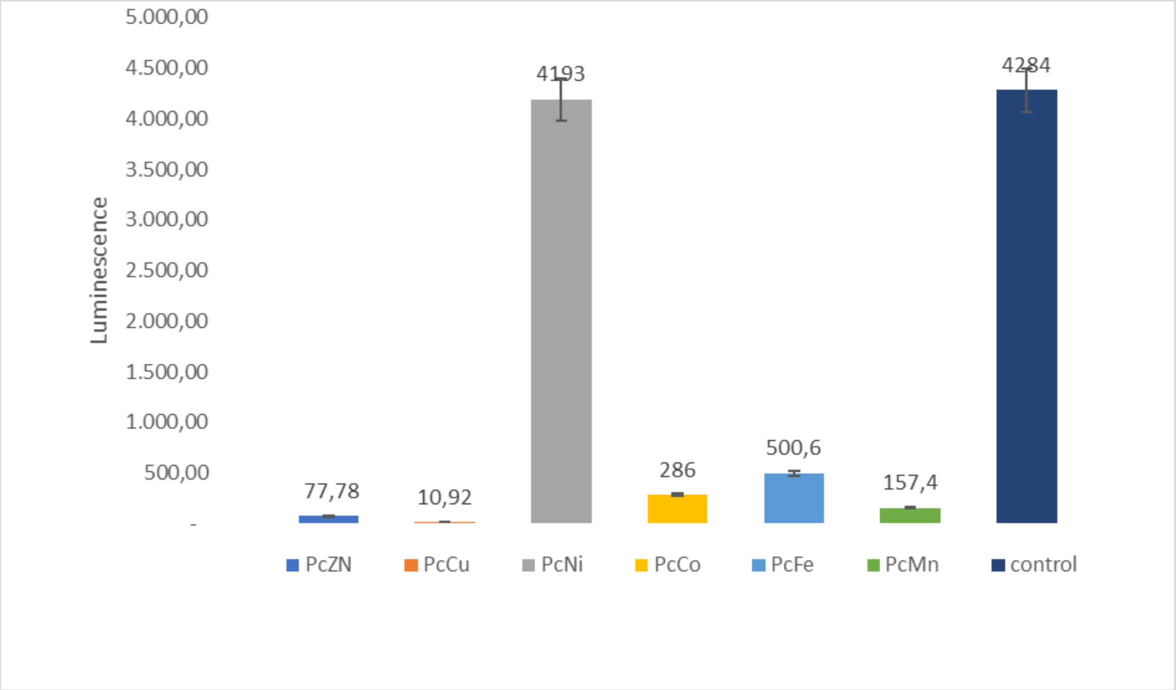

Figure 6. Bar graph of chemiluminescence of irradiated phthalocyanines in DMF, in the absence of superoxide dismutase (SOD). (control = H_2O_2 + luminol) $p < 0.05$ vs. control.

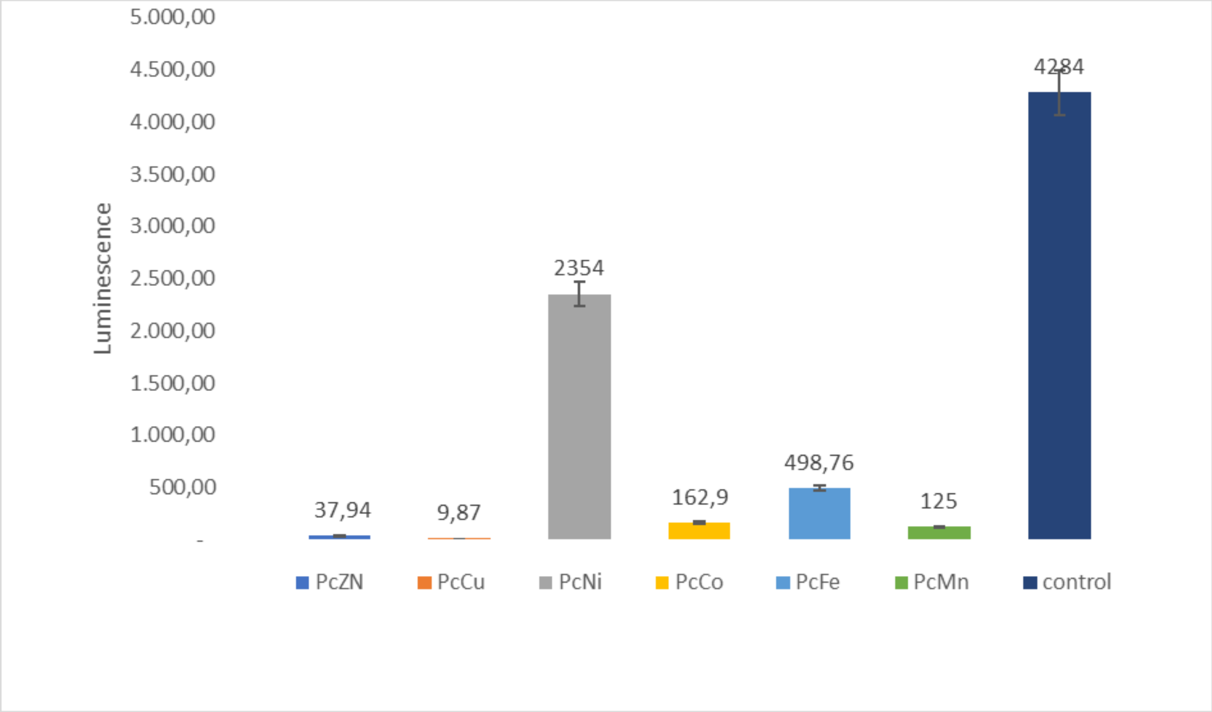

Figure 7. Bar chart of chemiluminescence of irradiated phthalocyanines in DMF, with the presence of superoxide dismutase (SOD). (control = H2O2 + luminol) $p < 0.05$ vs. control.

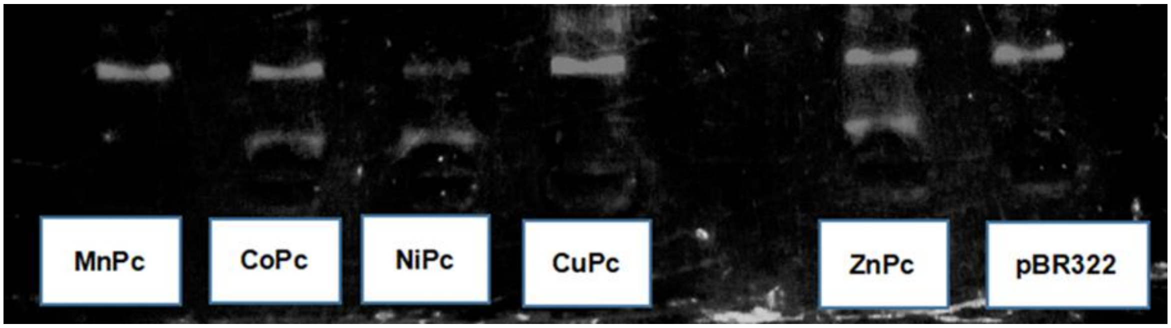

Figure 8. Photoactivity of phthalocyanines linked to different metals (Mn, Co, Ni, Cu and Zn, 0.01 M after being irradiated for 30 min) analyzed by the agarose electrophoresis technique.

4. Discussion

The trend in superoxide anion generation observed in the tests carried out in this study were as follows: PcNi > PcCo > PcZn > PcFe > PcCu > PcMn. This findingis consistent with the fact that phthalocyanines with a lower capacity to trigger the type II mechanism tend to "prefer" type I mechanisms, that is, they are more prone to electron exchange with triplet oxygen to photogenerate free radicals such as superoxide anion. This is mainly because they are phthalocyanine molecules with less time and energy capacity to photoinduce the generation of singlet oxygen through the type II mechanism. Furthermore, the low photogeneration of the superoxide anion radical by phthalocyanines bound to metals, such as Fe, Cu and Mn, can be attributed to the paramagnetic characteristic of these molecules which produces excited states with shorter lifetimes. The more pronounced effects in PcZnmay be due, for example, to its broad capacity to bind to the human erythrocyte membrane, in addition to its effective photosensitizing capacity to trigger both type I and type II mechanisms. In general, it can be said that all the phthalocyanines studied exhibited an effect on the bacterial plasmids pBR322, except for PcMn, which did not show appreciable activity on these molecules. This low effectiveness may be due to the low capacity of PcMn to photogenerate oxygenated free radicals through type I and type II mechanisms. the fact that this molecule is highly unstable under the conditions generally found in photodynamic applications. The insertion of different metals of the first transition series (Zn, Cu, Ni, Co, Fe and Mn) has a fundamental effect on the photochemical and photophysical properties of phthalocyanines, which “modulates” the photosensitizing characteristics of these molecules.

The high quantum yields of singlet oxygen in PcZn can be explained not only by its diamagnetic nature, but also by the possible influence of the spin-orbit coupling effect, which, on the one hand, favors crossing between systems and, consequently, leads to a greater population of molecules in the triplet excited state. In fact, computational calculations have reported a spin-orbit effectiveness value of 1.47 for the PcZn molecule, which may be sufficient for the population of triplet excited states and, therefore, generate singlet oxygen in a photoinduced manner much more effectively than PcCu, PcNi, PcCo, PcFe, and PcMn. Additionally, the singlet-triplet energy difference calculated for PcZn was approximately 1.04 eV, which is sufficient to generate singlet oxygen throuh energy transfer with the ground-state oxygen [20, 21]. On the other hand, the trend in superoxide anion generation using the NBT test was as follows: PcNi>PcCo>PcZn>PcFe>PcCu>PcMn. This finding is consistent with the fact that phthalocyanines with a lower capacity to trigger the type II mechanism tend to "prefer" type I mechanisms, that is, they are more prone to electron exchange with triplet oxygen to photogenerate free radicals such as superoxide anion. This is mainly because they are phthalocyanine molecules with less time and energy capacity to photogenerate singlet oxygen through the type II mechanism. Furthermore, the low photogeneration of the superoxide anion radical by phthalocyanines bound to metals, such asFe, Cu, and Mn, can be attributed to the paramagnetic characteristics of these molecules which produces excited states with shorter lifetimes [11, 13, 22]. Regarding PcNi, it can be stated that it is a photosensitizer that prefers the type I phototoxic mechanism with a very high efficiency.In addition, it is capable of producing singlet oxygen in solution possibly due to its own tendency to form aggregates, as previously discussed. Thus, it can be deduced that PcNi can generate phototoxicity through both type I and II mechanisms, which is consistent with the results previously obtained in our laboratory regarding the photosensitizing characteristics of Ni(II) porphyrins. These observations are based not only on the results of the NBT test, but also on the chemiluminescent light intensities revealed for PcNi, which appeared to be the phthalocyanine molecule with the hihgest capacity to photogenerate H_2O_2 in this study. For its part, the photogeneration of free radicals by PcZn, whether performed using the NBT or chemiluminescence assays, is consistent with in the findings of other studies.These studies highlight the high reducing capacity of this class of molecules, which is essential for promoting type I phototoxic mechanisms [23, 24]. On the other hand, the results of chemiluminescent light intensity in the presence of SOD indicate that it is inhibited by certain phthalocyanines. This is especially true for PcNi, where the chemiluminescent light intensity was reduced by almost 50% compared to assays performed without SOD. This indicates that PcNi has a stronger tendency to form the superoxide anion radical. A similar trend was oserved in PcZn, PcCo, and PcMn molecules, where the chemiluminescent light intensities were considerably reduced in the presence of SOD. However, in the particular case of PcFe, the intensity of chemiluminescent light was not reduced in the presence of SOD, which may indicate the involvement of other hydroxyl-type free radicals. The results of the photohemolysis procedures carried out show strong activity of PcZn on human erythrocytes, which is consistent with previous studies indicatin that this activity may be due to a large number of complex events developing at the cellular level as a result of the photosensitization process, such as cell swelling (injury), a reduction in the negative charge of the erythrocyte membrane surface and, cell aggregation. All of these are the result of the binding and interaction of the photosensitizer molecule with the human erythrocyte membrane, which is a necessary condition for the photoinduced hemolysis process to occur [17]. ideas According to these findings, the greater photohemolytic capacity of PcZn shown in this study can be attributed to its strong interaction with the membrane of the human erythrocyte, which makes the damage process induced by photosensitization (generation of type I and type II radicals) more feasible and effective.

5. Conclusion

Metallophthalocyanines, particularly those containing Zn and Cu, demonstrate significant potential as efficient photosensitizers for photodynamic therapy. Their ability to generate reactive oxygen species and induce DNA damage suggests promising applications in targeted cancer treatment. Further investigation into their in vitro and in vivo bioactivity will support the development of more effective PDT agents. The production of reactive oxygen species by irradiation of metallized phthalocyanines at optimal and efficient wavelengths is a significant and useful advance in photodynamic therapy. This work establishes the in vitro mechanisms of action for their potential applications in the treatment of cancer cells, as well as the phototoxic effects they can produce in human erythrocytes.

Ethical Considerations

Compliance with ethical guidelines

Not applicable

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Author's contributions

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Conflict of interest

The authors declare no conflict of interest.

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