Protoporphyrin IX: An Endogenous Theranostic Compound

Lilia Coronato Courrol
Departamento de Física
Universidade Federal de São Paulo
Diadema, São Paulo, Brazil
lcourrol@unifesp.br

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***Abstract*— Porphyria, cancer, and atherosclerosis patients manifest increased concentration of protoporphyrin IX (PpIX) in tissues and blood, and PpIX fluorescence can be used to diagnose these diseases. This review will describe the role of PpIX inside the cells and organisms. Diagnosis and therapy approach using PpIX will be described. Finally, we will also evaluate if PpIX could be used to diagnose and treat Covid-19 since an abnormal phenomenon related to hemoglobin dysfunction was observed in the patients with this disease.**

Keywords— Hemoglobin, Protoporphyrin IX, Fluorescence, Cancer, Covid-19

I. INTRODUCTION

Protoporphyrin IX (PpIX) is an organic compound that plays an important role in living organisms, animals, and plants, as a precursor of essential compounds like hemoglobin and chlorophyll[1].

PpIX is composed of four pyrrolic rings linked by methylene bridges (Figure 1). The PpIX tetrapyrrole structure enables the chelation with transition metals, for example, iron, magnesium, and zinc allowing the formation of metalloporphyrins, which perform a variety of biologic functions. In the animals and some microorganisms, PpIX is encountered in the form of complexes where the two inner hydrogen atoms are replaced by an iron (II) (ferrous) cation Fe^{2+}, in the heme structure (Figure 2)[2]. Hemes are prosthetic groups in some important proteins. These heme-containing proteins include hemoglobin, myoglobin, and cytochrome c. In plants, algae, and some bacteria, chlorophyll is synthesized with the insertion of magnesium into the tetrapyrrole macrocycle to make magnesium protoporphyrin IX [3] (Figure 2).

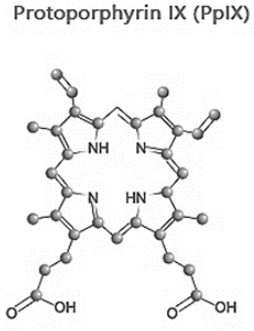

Figure 1. Protoporphyrin IX

Several organisms from bacteria to mammals can synthesize PpIX from basic precursors such as glycine and succinyl-CoA, or glutamic acid. In the early steps of biosynthesis (Figure 3), which starts from glutamic acid, a tetrapyrrole is created by the enzymes which transform aminolevulinic acid via porphobilinogen and hydroxymethylbilane to uroporphyrinogen III [4]. The next intermediates are coproporphyrinogen III and protoporphyrinogen IX, which are oxidized to the protoporphyrin IX. The metal cation is inserted into PpIX by enzymes called chelatases. In chlorophyll biosynthesis, the enzyme magnesium chelatase converts PpIX into Mg-protoporphyrin[4].

A. PORPHYRIN OPTICAL PROPERTIES

PpIX is a fluorophore. The absorption spectrum has five bands: Soret band (the most intense band), in the region of 400 nm, four Q bands, which comprise the region between 450 and 750 nm. The emission is observed in its two characteristic bands at approximately 635 nm and 705 nm (Figure 4a)[5].

Several types of chlorophyll exist in the photosystems. Chlorophyll a and b absorption and emission bands are shown in Figure 4b.

Porphyrins, metalloporphyrins, coproporphyrin III, and uroporphyrin III absorption and emission peaks and fluorescence lifetime are described in table 1.

TABLE I. TABLE 1. ABSORPTION EMISSION AND FLUORESCENCE LIFETIME OF PPIX COMPLEXES.

Fluorophore	Absorption (nm)	Emission (nm)	t (ns)
PpIX $C_{34}H_{34}N_4O_4$	**403**	**632 and 700**	**11.2**
PpIX-Zn $C_{34}H_{32}N_4O_4Zn$	**418**	**590 and 630**	**2.07 [6]**
Chlorophyll a $C_{55}H_{72}O_5N_4Mg$	**418**	**671 and 720**	**5.7[7]**
Chlorophyll b $C_{55}H_{70}O_6N_4Mg$	**453**	**644 and 700**	**3.7**
Uroporphyrin III $C_{40}H_{44}N_4O_{16}$	**397**	**618 and 682**	**4.3 [8]**
Coproporphyrin III $C_{36}H_{38}N_4O_8$	**389**	**614 and 674**	**[9]**

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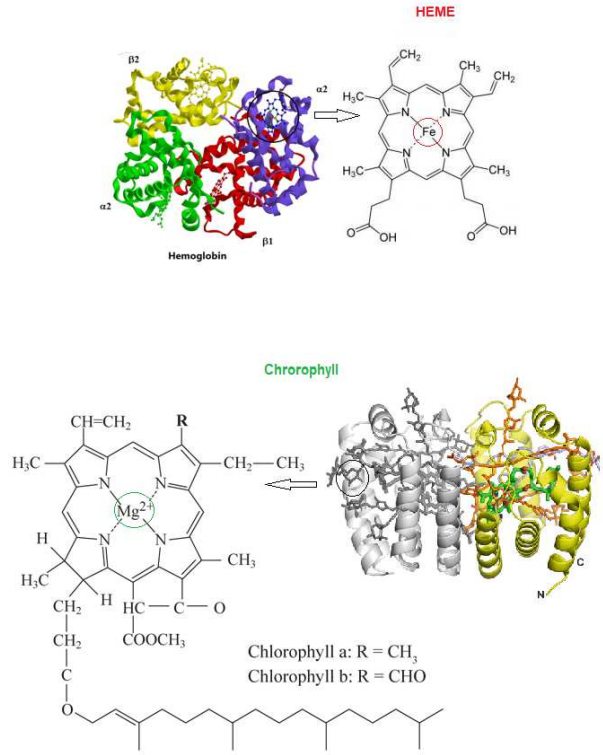

Figure 2 –Heme and chlorophyll structures. The top image shows the hemoglobin structure with the alpha chains (α1, α2), the beta chains (β1, β2), and the four heme groups and the structure of iron protoporphyrin IX subunit of heme B. Bottom image shows structural formula of a and b type chlorophylls.

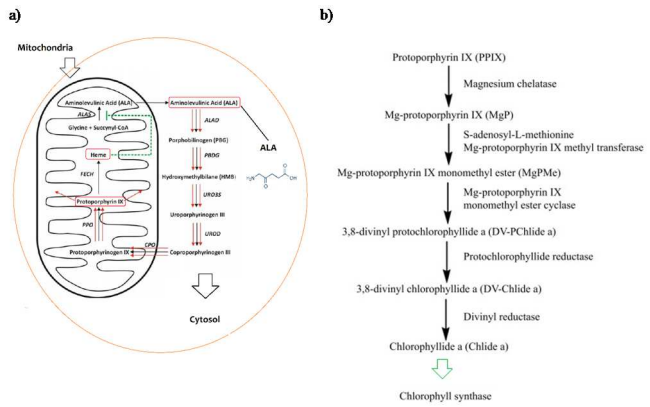

Figure 3. Heme and Chlorophyll synthetic pathways: a) In animals, insects, fungi, and protozoa, as well as the α-proteobacteria group of bacteria, the committed step for porphyrin biosynthesis is the formation of δ-aminolevulinic acid (δ-ALA, 5-ALA) by the reaction of the amino acid glycine with succinyl-CoA from the citric acid cycle. b) In plants, algae, bacteria (except for the α-proteobacteria group), and archaea, it is produced from glutamic acid via glutamyl-tRNA and glutamate-1-semialdehyde. The enzymes involved in this pathway are glutamyl-tRNA synthetase, glutamyl-tRNA reductase, and glutamate-1-semialdehyde 2,1-aminomutase.

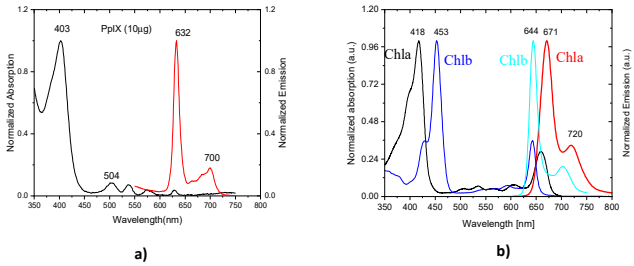

Figure 4: Absorption (a) and emission (b) spectra of PpIX (in acetone) and chlorophyll a and b (in methanol).

B. PpIX Fluorescence and Reactive Oxygen Species Generation

Fluorescent porphyrins that include PpIX, uroporphyrin III, and coproporphyrin III absorb light energy and undergo intersystem crossing with oxygen to produce reactive oxygen species (ROS)[10]. This is the principle of photodynamic therapy (PDT). The same process occurs if porphyrins are irradiated by ultrasound. In this case, resulted therapy is named sonodynamic therapy or SDT[11]. Both light and ultrasound can excite porphyrins from the ground state (S_0) to the excited singlet state (S_1) as illustrated in Figure 5. Porphyrins can then either relax back to S_0 state emitting light or go to the excited triplet state (T_1) by intersystem crossing. The energy of porphyrins in the T_1 state can either be relaxed by phosphorescence or can be transferred to ambient molecules by reactions type I and type II. In type I reaction, an electron transfer process produces free radicals, which further interact with water or oxygen molecules leading to the production of hydroxyl radicals (OH·) or superoxide anions (O_2^-). In the type II reaction, an energy transfer process between the T_1 state of porphyrins with $^3O_2$ results in the formation of highly cytotoxic singlet oxygen ($^1O_2$). The ROS can react with proteins, lipids, and nucleic acids, causing their oxidation and, consequently, damage to cells, tissues, and microorganisms as SARS-CoV-2 [12].

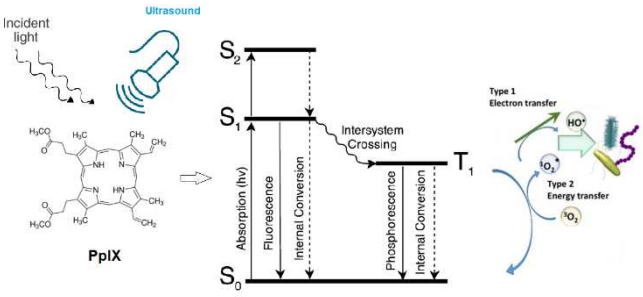

Figure 5. Schematic representation of type I and type II reactions following PpIX activation upon light or ultrasound excitation.

C. PpIX in Cell, Blood, and Tissues

Cells, tissues, bacteria, plants have porphyrins in their constitutions and for this reason, porphyrins are one of the most important fluorophores of biological material.

Figure 6 shows a calibration curve of synthetic protoporphyrin IX (Sigma). It is possible to use this curve to obtain quantities of PpIX extract for organs, cells, tissues, and blood from humans and animals as mice and rabbits or plants as the spectra as shown in figure 7.

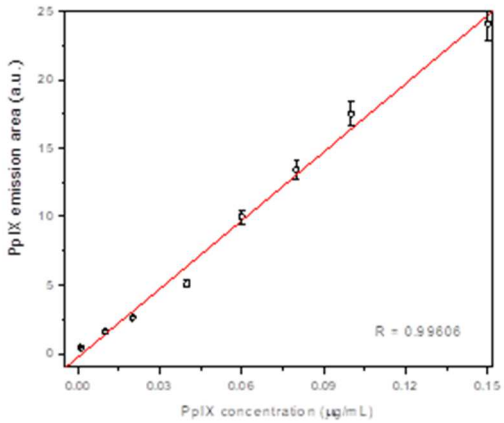

Figure 6. Increasing the concentration of PpIX in a solution prepared with acetone is possible to see an increase in the PpIX fluorescence band area (600-750 nm).

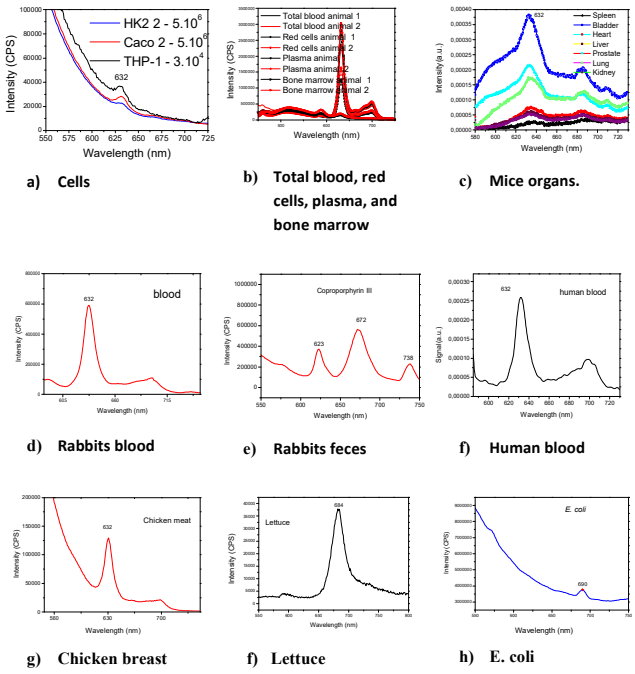

Figure 7: Fluorescence of PpIX extracted from a) cells-In black THP-1 (macrophages), in blue HK-2 (human kidney), in red Caco-2 (Human colon adenocarcinoma cell), b) Total blood, red cells, plasma, and bone marrow of two different mice, c) mice organs,spllen, bladder (dark blue), heart (light blue), liver (yellow), prostate (red), lung and kidney (green). d) Rabbit's blood, e) Rabbit's feces, f) human blood, g) chicken breast, f) Lettuce, h) E. coli. PpIX extracted with acetone. Spectra obtained exciting at 405 nm and the emission spectra shown in the figures were obtained from averages of n=3.

D. PpIX AND DISEASES

Most porphyrin production occurs in the bone marrow and liver. Porphyrins are transported from the biosynthetic source to target tissues through blood by binding to porphyrin binding proteins such as serum albumin. Cellular porphyrin levels are modulated by porphyrin transporters.

Atherosclerosis and cancer are considered the main causes of death all over the world. They share several important molecular pathways and many processes from the very early stages of development up to the advanced forms in both pathologies. Factors as inflammatory processes, uncontrolled cell proliferation, and oxidative stress are present in both diseases. Rapidly proliferating tissues, like cancer and atheromas, may preferentially accumulate porphyrins[13].

Figure 8 presents a curve obtained for PpIX emission spectrum area between 575 and 725 nm as a function of the growth of the induced prostate tumor (inoculated with DU145 cells) in nude mice. Each point corresponds to the mean of the signal of each studied group. The PpIX was extracted from the blood animals healthy (control group CG) and with tumor, in the 7th, 14th, 21st, 35th, and 49th day after the inoculation procedure. For the tumor group, the animals had blood collected in the seven weeks following tumor induction (on the same days as the CG group). It could be observed that the mean intensity value of the tumor group increases progressively as the tumor grows within the animals, indicating that porphyrin is accumulated in the blood[14].

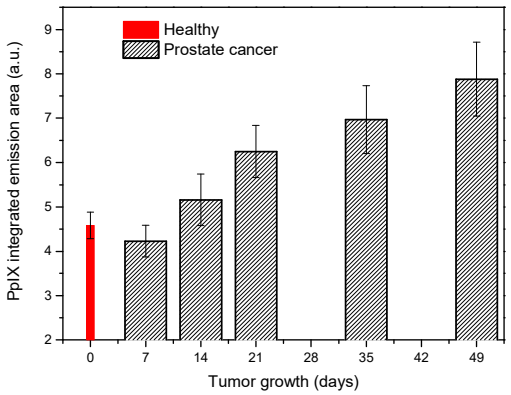

Figure 8 – Area (between 575 and 725nm), of the PpIX emission spectrum extracted from the blood of nude mice, plotted as a function of the tumor growth days. Day 0 corresponds to the Control group, and days 7, 14, 21, 35, and 49 indicate the emission intensity of endogenous PpIX in the animals of the tumor group.

Rabbits fed with 1% cholesterol were investigated for their PpIX total blood accumulation response. For this purpose blood porphyrin was extracted from animals, in which the atheroma plaques were induced by high cholesterolemic food administration. The results are shown in Figure 9. In this figure, the signal emission area obtained integrating endogenous PpIX emission spectra in the range of 575–725 nm, was plotted as a function of days of plaque growth (20th, 40th, 60th, 82th, and 89th days after diet procedure administration). Each point corresponds to an average of signal from each studied group (3 animals/group). It can be observed that the mean of the values increases in intensity as

the arteries grow, indicating that porphyrin is accumulating in blood[15].

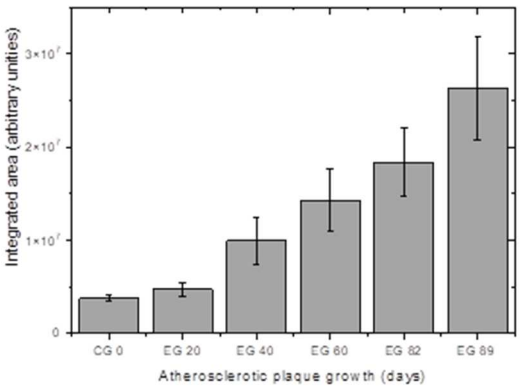

Figure 9. The area of emission spectra of PpIX extracted from animals' blood, in the range of 575–725 nm plotted as a function of the days high cholesterol diet administration). Day 0 corresponds to Control Group and Days 20, 40, 60, 82 and 89 correspond to days of diet.

So, abnormal increase in PpIX fluorescence in blood can predicte diseases as cancer and atherosclerosis.

PpIX accumulation mechanism and the difference between high and low accumulation in rapidly proliferating tissues remain unclear. It was observed that ABCB6 transporters upregulation played a key role in PpIX accumulation in tumor cells[14].

Genetic porphyrias comprise eight diseases caused by a defect in a specific enzyme of the heme biosynthetic pathway, leading to the accumulation of heme precursors[11]. Accumulation of PpIX in human porphyrias can cause skin photosensitivity, biliary stones, hepatobiliary damage, and even liver failure[12].

Porphyrins bind to native proteins and, in presence of light and oxygen, oxidize several amino acids, particularly methionine[16]. This explains the acute photosensitivity in most porphyrias. However, light-induced porphyrin-mediated oxidative stress does not account for the effect of porphyrins on internal organs. In internal organs, light-independent porphyrin-mediated protein aggregation occurs after secondary triggers of oxidative stress. The organ oxidative stress may derive from inflammation or other insults. This, in turn, leads to the formation of ROS and protein oxidation. Porphyrins then bind to the oxidized proteins further promoting aggregation which is a major mechanism of cellular injury in porphyria. [16].

E. PPIX AND PLANTS

PpIX and Mg-PpIX optical properties are especially significant in the case of photodynamic herbicides [17].

Plant photodynamic stress is considered as abiotic stress linked to ROS production as the first cause of cell death [18]. Porphyrins could be relevant to reduce the use of pesticides while maintaining high yield as well as high quality in agricultural production. Porphyrins are photodegradable and non-toxic under dark as well as they were used at micromolar concentrations.

Lead and mercury inhibited porphyrin biosynthesis. Chlorophyll a and chlorophyll b and total chlorophyll contents in dark-grown seedlings significantly decreased, suggesting the impairment of chlorophyll biosynthesis by lead and mercury in germinating seedlings[19]. So, a decrease in the chlorophyll emission band can indicate the presence of lead or mercury in soil.

Concerns about the pesticide residues in agricultural products have been raised in recent years because of increasing health concerns and public interest in food quality and safety[20]. Thus, rapid, convenient, and accurate analytical methods for the detection and quantification of pesticides are urgently required. Recently, the aggregation effect of pesticides on the porphyrins gold nanoparticles was successfully applied for the rapid and accurate determination of multicomponent pesticide residues (Paraquat, Dipterex, Dursban, methyl thiophanate, and Cartap) in food samples by observing changes in the porphyrin (422 nm) UV-vis spectra[20].

F. PPIX AND BACTERIA

The microbiological contamination causes concern to public health by both the pathogenic action of microorganisms and the increasing antimicrobial resistance observed in bacterial strains.

Iron is an important cofactor in many biological systems, and it is necessary for respiration in bacteria[21, 22]. Pathogens have developed strategies to scavenge heme from hemoglobin or myoglobin extract the iron from heme.

In odontology, several publications report the detection of oral biofilm by fluorescence when the teeth are illuminated with light in the blue spectral region [23].

Considering that most microorganisms and animal cells excrete porphyrins, the evaluation of porphyrin contents in meat by fluorescence spectroscopy was proposed. The presence of some microorganisms in meat can be correlated by an enhancement in PpIX fluorescence intensity.

Figure 10 shows PpIX fluorescence spectra obtained from chicken meat contaminated with *E. coli*, *Salmonella,* and *Campylobacter* in comparison to signal obtained from uncontaminated meat[24]. DyP peroxidases, identified in *E. coli*, facilitate the release of iron from heme, preserving the tetrapyrrole ring, generating a free iron cation and PpIX. *Salmonella* liberates coproporphyrin III instead of PpIX. PpIX fluorescence extracted from meat contaminated with *Campylobacter* was comparable to non-contaminated meat[24].

G. PPIX AND VIRUS

SARS-CoV-2 infectivity in erythroid progenitor cells was reported[25]. SARS-CoV-2 infection either directly or

indirectly induces stress erythropoiesis. Intensive care COVID-19 patients suffer a decline in hemoglobin levels and an increase of circulating nucleated red cells[26].

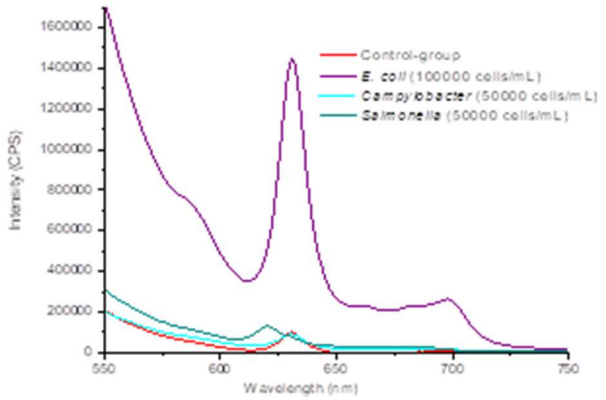

Figure 10. Chicken meat with 100 µL of suspension of *E. coli* (10^5cells/mL), *Campylobacter* (50000 cells/mL) and *Salmonella* (50000 cells/mL) or 100 µL of PBS (control group – uncontaminated) stored at 25 °C for 48 h. Porphyrin fluorescence spectra obtained from the meat with excitation at 410 nm.

SARS-CoV-2 that depends on iron to replicate within living host cells, obtains this iron at the expense of hemoglobin and heme, which once disrupted, release PpIX[27]. An abnormal accumulation of porphyrins is associated with severe Covid-19 [26].

The analysis of the intrinsic PpIX fluorescence from blood, urine, and feces samples can yield information on the presence of SARS-Cov-2 in the body[28].

It is important to mention that porphyrins when excited by the light, like sunlight, with sufficient intensity, can effectively destroy SARS-CoV-2 by photobiomodulation or photodynamic process depending on the level of ROS generated in the process [29]. The penetration of the optical radiation into the biological tissue with wavelengths around 400 nm, the maximum of the porphyrins absorption bands, is around ~250 nm [30] and for this reason, the only superficial effect can be obtained. Nevertheless, sonodynamic therapy could be employed to eliminate SARS-CoV-2 in deeper regions as the lungs[31].

II. Conclusions

Porphyrins have been shown in several different studies to be a promising class of compounds to use for diagnosis and therapy approaches. This paper presents novel insights into the porphyrin content in tissues, cells, bacteria, viruses, and plants and the connection between porphyrin content and diseases.

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