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Synthesis and Characterization of an Europium-Porphyrin Complex

Research Project Presented to the faculty of the Governors State University College of Arts and Science Chemistry Department

In partial fulfillment of the requirement for the Masters in Science in Analytical Chemistry

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Abstract

Photodynamic therapy (PDT) is a promising treatment that has continued to improve over the past thirty years when the first commercially approved photosensitizer (PS) was introduced. Although PDT has many successful applications, the terrifying number of new cancer cases reported each year makes scientists focus their efforts towards the development of new efficient PS for PDT. The biggest obstacle that prohibits PDT from becoming a more widely used therapy is the ineffective photosensitizers (PS) that are available on the market today. The purpose of this study was to evaluate the synthesized metal-porphyrin complex as a possible candidate as a PS. The metalloporphyrin was synthesized from the combination of protoporphyrin and europium. The experiments performed in this research to evaluate the new drug were spectroscopic measurements, DNA photocleavage studies, phototoxic tests, and DNA binding. The results obtained provide this new drug, which is in its initial developing stages, with great future potential in the PDT field.

I. Introduction
According to the National Cancer Institute, there was about 1.5 million new cancer cases in the United States in 2009 [6]. The most frequent procedures to treat cancer are chemotherapy, radiation therapy or surgery. Although these treatments have been proven effective they came attached with a high health risk due to their harmful side effects. The scientists has been looking for more noble therapies that do not involve the life threatening side effects that chemotherapy, radiation therapy, or surgery have. Among the new treatments available, Photodynamic therapy (PDT) has gained much attention in the past 30 years with the introduction of the first commercially available drug Photofrin® (Porfimer Sodium) and the publications of the successful treatment of lesions using PDT by Dougherty et al [3]. Since then, PDT has shown great potential for diverse types of cancer with only mild side effects. For example, PDT using the drug Porfimer Sodium (Photofrin®) was approved for the treatment of oesophageal cancer in the United States, for the treatment of early and late stage lung cancer in the Netherlands, bladder and oesophageal cancer in Canada, and lastly, for early stage lung cancer, oesophageal cancer, gastric cancer, and cervical cancer in Japan [1].

One of the most appealing aspects of PDT is that it is a minimal invasive treatment and it has low side effects [8]. PDT uses a photosensitizing drug, also called photosensitizer (PS), to transfer energy from a specific source of light to cellular oxygen to create reactive oxygen species and induce cell death [7]. Since the drugs available on the market today for PDT use are not very effective, a high concentration of the drug is needed to produce an acceptable phototherapeutic response. Although photosensitizers (PSs) are more selective towards tumors than healthy tissue [7], the drug normally ends up accumulating in cutaneous tissue leaving patients with skin photosensitivity [6][14]. Consequently, patients in PDT treatment have to avoid the exposure to direct sunlight for several days (normally at least 2 to 3 weeks [1][4]) in order to prevent sunburn [2]. Scientists have seen that one of the major barriers that PDT has is the poor effective drugs and therefore, there is much effort today in the PDT investigational field to produce second and third generation drugs that are more effective and selective than the first generation PSs [4].

The purpose of this study was to develop and evaluate a porphyrin derivative complex as a possible photosensitizer for PDT. The PS was synthesized in-house using protoporphyrin as a ligand and inserting the lanthanide europium to the center of the tetrapyrrrole macrocycle. The porphyrins that are in the market such us Photofrin and other like sensitizers have weak
absorbances in the optimal phototherapeutic region with the absorbing band at around 630nm \cite{12}. It is known that metal ions that are attached to porphyrins change the photophysical properties of the macrocycle providing the new molecule with innovative characteristics \cite{11}. The insertion of the Europium in the center of the macrocycle was used to enhance the absorption properties of the new synthesized metal complex. In order to evaluate the porphyrin-lanthanide complex effectiveness as a PS, absorbance and fluorescence spectroscopy studies, DNA Biding studies, DNA photo cleavage studies, and median lethal dose (LC$_{50}$) cell studies were performed. Even though it was understood that the new complex is in the first stages of development the studies performed in this research provided with the basic necessary information needed to understand the possible efficiency of the synthesized compound as a photosensitizer.

The characteristics desired in a PS to be consider effective are to be selective towards the target tumor, low side effects, chemically pure, low dark toxicity and high toxicity when activated, absorption wavelength in the red visible region from 600nm to 800nm for deep tissue penetration of the incident light, easily available, and low cost \cite{9,12}. Generally, PSs come from three main families, porphyrins, chlorophyll derivatives, and dye substances \cite{9}. The mechanism of energy transfer employed by the PS can be followed by the Jablonski diagram in figure 1. First, the PS is excited by a specific source of light from its ground state ($S_0$) to a short live singlet state ($S_1$). Then, from the $S_1$ it can return to the $S_0$ by radiative and non radiative decay. During non radiative decay the PS undergoes intersystem crossing (ISC) producing a longer lifetime triple state ($T_1$). From $T_1$ the PS goes back to $S_0$ as phosphorescence where it can also transfer energy to other molecules present in the environment by a type I or type II reaction. In a type I reaction, electron transfer from the PS produces free radicals which they react with cellular oxygen producing superoxide and peroxide oxygen anions. In a type II reaction, the PS directly transfers energy to molecular oxygen from the excited $T_1$ to triplet ground state oxygen producing reactive oxygen species. The type I or type II reaction triggers oxidative stress on the cell producing cellular death \cite{8,1}.

The synthesized metalloporphyrins showed some of the desirable characteristics in good PS. However, the main purpose of this study was to provide with the basic studies to support the synthesized marocyclic molecule in its initial stage of drug development. There are many futures that can be later added to the molecule to improve its selectivity toward cancerous tissue, its intermolecular localization, and its effective delivery to targeted tissue.
II. Materials and methods

II.I. Synthesis and purification of the Metalloporphyrin

The synthesis was carried according to literature. The Protoporphyrin 95% was purchased from Sigma Aldrich with a lot P8293-G. Shortly, Europium oxide was combined with protoporphyrin and deionized (DI) water in a round flask. Reflux was conducted for 72 hours under 90°C to 100°C. The product was washed with DI Water, filtered, and dried. The compound was purified by column chromatography using the size exclusion bed Sephadex G-15.

II.II. Spectroscopic Studies

To obtain the absorbance spectrum of the metalloporphyrins and the protoporphyrin, both compounds were dissolved in dimethyl sulfoxide (DMSO) to a concentration of 200µM. The
ultraviolet and visible (UV-VIS) spectrum of the compounds was measured using an Ocean Optics USB4000 spectrometer, refer to Figure 4. The fluorescence spectrum of the metalloporphyrin complex and the ligand was measured using an Ocean Optics USB2000+ spectrometer (spectrums can be observed in figure 4). Both compounds were diluted to a concentration of 100µM. The infrared (IR) spectrum for the metalloporphyrins and the protoporphyrin was acquired using a Thermo Fisher equipped with an attenuated total reflectance (ATR) accessory (refer to figure 3). Both compounds were dissolved in DMSO to a total concentration of 200µM.

II.III. DNA binding studies

For this study a titration assay method was employed to calculate the DNA binding constant. Briefly, the metalloporphyrins was dissolved in DMSO to a total concentration of 100µM. The absorbance of the solution was measured at intervals of 5 minutes with the addition of 10µL of CT-DNA using an Ocean Optics USB4000 spectrometer. The following equation was used:

\[ \frac{\varepsilon_a - \varepsilon_f}{\varepsilon_b - \varepsilon_f} = \frac{1}{K_b [DNA]} + 1 \]

To Produce the Stern-Volmer plot and calculate the Stern-Volmer constant the same titration method was used as described above for the DNA binding constant but instead of the absorbance the fluorescence of the solution was measured using an Ocean Optics USB2000+ spectrometer. The following equation was used to calculate the Stern-Volmer constant:

\[ \frac{F_o}{F} = 1 + K_{sv}[Q] \]

II.III. DNA Photocleavage Studies

The agarose gel was prepared in house with a concentration of 1.4%. 10µL of plasmid DNA and 10µL of the metal-porphyrin complex were one hour and then photo-irradiated for one hour. The dye was added to the mixture and then the mixture was loaded into the agarose gels wells. Electrophoresis was performed for 80 minutes at 70v.

II.IV. LD\textsubscript{50} Studies

The cells were growth with an enrichment media. The media was washed off with PBS and the Drug was added. The cells were incubated for a few hours and then the cells were reacted in a dark and lighted environment. The drug was washed away and enrichment media was added to
the cell cultures. The plates were incubated until the control group reached an 80% concentration. The media was washed off and the cells were burst with a detergent solution. At this point the albumin standard solution was added and incubated for few hours. A plate counter was used to quantify the cells.

III. Results and Discussion

III.I. Synthesis

The synthesis proceeded as the literature indicated and the molecular structure of the synthesized metalloporphyrins can be viewed in figure 2. The results gained during the spectroscopy studies confirmed the successful insertion of the metal in the tetapyrrolic macrocycle. The IR spectrums form the ligand and the metal-porphyrin complex showed a hypsochromic shift for the band at around 1000 cm$^{-1}$, which can be observed in figure 3. The IR spectrum of the metalloporphyrin had a band at 1018.2cm$^{-1}$ while the ligand exhibits the corresponding band at 1019.7cm$^{-1}$ in its IR spectrum. The same structural confirmation was observed with a red shift produced in the metalloporphyrins for three of the visible absorbance bands. The lambda maximum ($\lambda$ max) obtained for those three bands are 505.5nm, 539.8nm, and 575.1nm. The equivalent $\lambda$ max for the ligand were obtained at 503.5nm, 539.6nm, and 573.4nm; which can be seen in figure 4.
**III.II. Spectroscopic studies**

The UV-VIS spectrum obtained for the metal-porphyrin complex shows four distinct bands with $\lambda_{\text{max}}$ at 505.5nm, 539.8nm, 575.1nm, and 629nm; they can be seen in figure 4. The molar absorptivity ($\varepsilon$) calculated for these four $\lambda_{\text{max}}$ are $3670.0 \, \text{M}^{-1} \, \text{cm}^{-1}$, $2905.7 \, \text{M}^{-1} \, \text{cm}^{-1}$, $1916.0 \, \text{M}^{-1} \, \text{cm}^{-1}$, and $1345.8 \, \text{M}^{-1} \, \text{cm}^{-1}$ respectively. The UV-VIS spectrum for protoporphyrin provided with four bands with a $\lambda_{\text{max}}$ at 503.5nm, 539.6nm, 573.4nm, and 629nm. According to the literature the commercial PS Photofrin has a $\varepsilon$ of $1170 \, \text{M}^{-1} \, \text{cm}^{-1}$ [12]. The insertion of the metal in the tetapyrrolic macrocycle enhanced the absorption of the ligand providing the metalloporphyrin with a stronger $\varepsilon$ than a commercially available drug. This absorption improvement is also reflected in the fluorescence spectroscopic studies.

The Fluorescence spectrum gather for the protoporphyrin exhibited two bands with a fluorescence maximum at 631nm and 678nm (figure 4) and an intensity of 1895.6 counts and 3162.8 counts respectively. The fluorescence spectrum collected for the metalloporphyrins provided with two districts fluorescence maximum at 631nm and 678 nm, refer to figure 4. The fluorescence intensity for those two bands is 20273.76 counts and 5911.5 counts respectively. The enhance fluorescence by the metal insertion into the core of the ligand macrocycle coincides with the data obtained by the absorption spectroscopy studies. This can indicate a great potential for this new metal-porphyrin complex since it already shows some Photophysical properties better than the available drugs in the market.
Figure 4. The UV-VIS spectrum on the left corresponds to the metalloporphyrin in DMSO. The UV-VIS spectrum to the right belongs to the protoporphyrin in DMSO.
Figure 5. The fluorescence spectrum to the left belongs to metalloporphyrins. The fluorescence spectrum to the right corresponds to the protoporphyrin.

**III.III. DNA binding studies**

One resourceful way to induce cell death is to cleave DNA. Therefore, binding DNA properties of the PS are a good source to measure the photosensitizer’s potential efficacy in PDT. The binding constant ($K_b$) was obtained from figure 6 at $1.76 \times 10^3$ M$^{-1}$. The low $K_b$ calculated is thought to be due to the possible fact that the metalloporphyrin does not bind directly to the DNA. However, it binds indirectly with a distance enough to allow energy transfer. This energy transfer is supported by the Stern-Volmer plot generated using the DNA titration method.

![DNA Binding Studies Graph](image)

Figure 6. DNA binding constant graph, data obtained from the DNA titration assay.

The Stern Volmer plot is a good indicator for the intermolecular deactivation capabilities of molecules. In this case it was used to measure the quenching ability of the DNA to the excited metalloporphyrins. The data obtained for this study was plotted in a graph and the results can be observed in figure 7. The Stern-Volmer constant ($K_q$) acquired from the slope of the graph is $1.29 \times 10^4$ M$^{-1}$, which shows the good quenching capacity that the DNA towards the synthesized metal-porphyrin complex.
Figure 7. Stern-Volmer plot for the DNA binding assay.

**III.IV. DNA photocleavage**

The effectiveness of the synthesized molecule to cleave DNA can be seen in the results obtained for the DNA Photocleavage assay. From left to right wells number 1, 2, 3, 5, and 7 were loaded with DNA only and wells number 4, 6, and 8 were loaded with DNA; both gels were prepared the same way. The appearance of two bands on wells 4, 6 and 8 in both gels indicates that protoporphyrin can cleave DNA with or without the presence of a Europium in its molecular structure. Nevertheless, the wells 4, 6, and 8 in the protoporphyrin gel are stronger in appearance than in the metalloporphyrins gel. This indicates that Europium can aid in decreasing the toxicity of protoporphyrin.
III.V. $LD_{50}$ studies

The dark and light toxicity of the synthesized metalloporphyrins was evaluated with the $LD_{50}$ study. The data obtained was plotted in a graph, which can be seen in figure 9. The calculated LC50 from the graph is 500µM, which indicates that the metalloporphyrins has good toxicity when activated by light and low toxicity when is not activated in the dark. This is an important feature to PSs need to have in order to be voluble for PDT\textsuperscript{[12]}. 
A nonexistent efficient photosensitizer in the market for PDT creates a big urge in the scientific field for the search of the ideal PS. This was the purpose of this study and the results obtained provided useful and promising insight on the europium-porphyrin complex synthesized. The fact that the $\varepsilon$ obtained from the spectroscopy studies is greater than the Photofrin $\varepsilon$, which is a widely use PS in the PDT field, gives the metalloporphyrin already a good starting point.

An impressive point of this new synthesized molecule is its good phototoxicity and low dark toxicity showed in this investigation as well as its ability to effectively bind and cleave DNA. Although the synthesized drug is still in its first baby steps of development, there is a lot work done by other scientists with second and third generation PS that can enrich the future of this noble drug.

For example, second generation PS are characterized by the addition of chromophoric structures to existing drugs to improve their absorption to the far red visible region (650nm to 800nm)\cite{5}. This can allow the PS to be used in PDT and improve its ability to work in deep tissue areas. On the other hand, Third generation PS are known for their ability to be more selective towards cancerous tissue. Some scientists reported enhanced selectivity of the PS by the addition of tumor specific monoclonal antibodies\cite{13}. Lastly, the delivery of the PS to its final
destination cannot be ignored since it is an area highly studied by the nanotechnology field. Scientists are producing nonocriers that boost the therapeutic response by efficiently delivering the PS its final destination\[^1\].

V. References


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