SYNTHESIS AND APPLICATION OF PORPHYRINATED LATEX PARTICLES FOR
PHOTODYNAMIC THERAPY

by

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Abstract

Photodynamic Therapy is a cancer treatment that requires a photosensitizer to reach a tumor in order to be excited by electromagnetic radiation sources so that the cancer killing agents such as Singlet Oxygen can be generated. In this work, the photosensitizer Protoporphyrin IX (PPIX) is improved with PS4VP nanoparticles to exhibit better photosensitive properties in aqueous solutions. Improvements include spectroscopic properties like absorption and photoluminescence emissions. In vitro studies showed better cell uptake, dark toxicity and photodynamic action, killing up to 85% of PC3 prostate cancer cells. Typical PPIX characteristics including photo-bleaching and photomodification were also observed when nanoparticle variations were performed, where the photoproduct photoprotoporphyrin (PP) developed separate absorptions and emissions from PPIX. The rise of PP was also accompanied with singlet oxygen generation. After observing PPIX retaining properties and improved, the organic scintillator 2, 5-diphenyloxazole was added to the particle system and FRET was observed where PPO transferred energy from X-ray to excite PPIX and giving PPIX emission via X-ray.

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Chapter 1 Introduction

Cancer, also called malignant neoplasm, is the second leading cause of death in the United States of America. From 2014-2016, estimations of new cases has increased from 1,665,540 to 1,685,210 while estimations of cancer deaths has increased from 585,720 to 595,690.\textsuperscript{1,2} In contrast to benign neoplasm, which pose no threat, malignant neoplasm is a label for diseases that that involve abnormal and uncontrollable growth of cells which can lead to tumor masses and even metastasis, where cancer cells can invade other organs and tissues, In response to this and over time, numerous therapies have become employed, and more are being developed. These treatments, including surgery, chemotherapy, radiation therapy, and photodynamic therapy have respective advantages and side effects which are used, sometimes in combination, to do the most to relieve patients of their conditions and to make cancer the least of their problems.

One of the conventional methods of removing tumors is through surgery, depicted in Figure 1. Typically, as long as the tumor has not undergone metastasis, surgical removal of the cancerous tissues is the most direct way towards elimination. However, with the direct action of this invasive treatment, risks are prone throughout the procedure. Aside from patients’ preconditions, the local anesthesia, pain effects and infection due to incision can cause major complications during the procedure.\textsuperscript{3} In addition, allergic reactions to the anesthetics and other medicine used can cause low blood pressure, unwanted bleeding which can result in the need for blood transfusions, and sudden fatal damage to blood vessels and internal organs during surgery can cause further complications.\textsuperscript{4-6} In some cases where some cancerous tissues has infiltrated in surrounding normal tissues, it becomes more difficult to remove tumor masses completely. Fluorescent dyes have
mitigated a portion of this obstacle. These dyes help surgeons to see cancer tissue that
have localized away from a tumor. However, there are few dyes able to reveal full
delineations of tumors, which is still risky to any vital organs in the area, adding to the
drawbacks and obstacles previously mentioned.

Figure 1 Surgery

Aside from surgically removing cancer tissues, chemotherapy utilizes toxic anti-
cancer drugs to target cancer cells that grow rapidly, as shown in Figure 2. However, this
selectivity can also affect and damage normal cells that have a comparable rate of cell
growth. These can include hair follicles, the digestive track, the reproductive system, the
bone marrow, and organs such as the heart, brain, kidneys and liver. Some side effects
from the drugs on these systems are loss of hair, damage to the digestive system, anemia,
infections, fatigue, sores, and sperm cells.5-10
While chemotherapy can reach and affect cancer cells once the drugs are distributed throughout the body, radiation therapy, seen in Figure 3, can be more precise. Without using drugs, high energy photons are directly targeted to tumor sites to damage and/or destroy cancer cells. The drawback of treating with energy radiation lies in the dosage during the treatment. The energy of the radiation needs to be high in order to first penetrate the skin and tissues before even arriving at the tumor site with remaining sufficient energy for treatment. Also, while the cancer cells are destroyed, some DNA of normal cells in the path of the radiation beam may also be damage, which can lead to abnormal growth control or even secondary cancers.\textsuperscript{11-12}
Similar to the targeting factor from radiation therapy and yet different from conventional chemotherapy, drugs have been developed with targeting mechanisms to fight cancer in the form of targeted therapy. Many cancer cells have different receptors and also a different number of receptors in comparison to normal cells at the cell surface. Major processes that are targeted are angiogenesis, where new blood vessels develop to supply oxygen and nutrients to the cancer sites, and growth signaling, where cells are told to divide or grow uncontrollably. Targeted therapeutic drugs aim at the vascular endothelial growth factors (VEGF) to limit or halt angiogenesis and epidermal growth factor receptors (EGFR) to slow or shut down cancer cell division and growth. A few drawbacks lie in normal skin cells and normal small blood vessels because drugs targeting EGFR can also affect skin
cell and cause problems while drugs targeting VEGF can also affect the small blood vessels which can lead to diseases like Hand-foot syndrome.\textsuperscript{13-17}

Instead of introducing foreign agents to directly destroy cancer or cancer processes, the patients' own immune cells can be boosted to allow it to fight the cancer by using immunotherapy. Monoclonal antibodies are artificial immune system proteins that can attach to specific proteins in cancer cells. This high specificity saves normal cells from being affected. Some side effects, however, include headache, vomiting, diarrhea, nausea, low blood pressure, fatigue, fever, and chills. Another mode of immunotherapy called cancer vaccines help boost immune responses to preventing disease. In addition, they can prevent and treat cancer. Some effects from these vaccines include high blood pressure and difficulty breathing. A nonspecific approach to immunotherapy helps the immune system become more aware and able to fight cancer. Side effects due to this form of therapy include flu-like symptoms, abnormal heartbeat, low white blood cell counts, thinning of hair and chest pain. Although there are side effects, immunotherapy works to promote sufficient quantity of immune cells with a strong recognition of tumor antigens. These cells must be able to traffic to and infiltrate the tumor, and the immune cells must be activated at the tumor site for working mechanisms such as direct lysis or cytokine secretion to cause tumor destruction.\textsuperscript{18-20}

Despite having numerous side effects from each respective cancer treatments, combinations of treatments can overcome respective drawbacks to approach the end result of tumor removal.\textsuperscript{21} While disadvantages from these are seen post treatment, another type of unconventional cancer treatment called photodynamic therapy, shown in Figure 4, while having some side effects, which includes photosensitivity reactions and swelling leading to
pain, has major pre-treatment drawbacks. Specifically, the obstacles lie not only in the formulation and fabrication of the drugs for this form of treatment, but also the light source by the factor of reachability. Photodynamic therapy uses a combination of non-toxic drugs, typically photosensitizers, with light and oxygen to treat and kill cancer. Each of the three ingredients, when existing separately, can do little to no harm to normal cells, but, when properly combined, they are detrimental to cancer cells.

**Photodynamic therapy - drug with radiation**

![Diagram of Photodynamic therapy]

Figure 4 Photodynamic therapy

Briefly, light is used to activate and induce a reaction from the photosensitizer in order to transform oxygen into a cancer killing agent. The focus in improving this therapy then becomes ensuring not only that the light can activate the photosensitizer, but also the choice of light can still activate the photosensitizer after having travelled through the body.
up until it reaches the desired human site. Also, some treatments using photosensitizers have been limited to treating the skin. Consequently, in addition to the conditions for light propagation and to improve the usage for deeper tumor sites, the photosensitizer must be able to travel or be transported through the body. Photosensitizers, organic, inorganic, and combinations of materials such as gold clusters with chlorin e6, Protoporphyrin IX, and copper complexes are being researched, and each has respective capabilities and efficiencies in generating reactive oxygen species. Many photosensitizers can absorb and be excited by visible light, and treatments use ones that can absorb red light because red light can penetrate up to 3 um into tissue. Because of this, clinical treatment with photodynamic therapy has been majorly limited to topical areas such as the upper layers of skin.

In order to overcome these limitations and to further the applicability of photodynamic therapy, investigations into the components need to include the following: the improvement of photosensitizers for the biological application, the maintenance of photosensitizer properties after modifications, and extension of the photosensitizer's activity for deep tissue PDT. We propose using a nanoparticle to modify the photosensitizer Protoporphyrin IX to improve its effects in PDT, maintain its spectroscopic and chemical characteristics, and allow an energy transfer system to use X-ray activation. Chapter 2 will give further background information of the mechanism of photodynamic therapy, Protoporphyrin IX, and some instrumentation used.
2.1 Luminescence

Typical photosensitizers emit a lower energy of light (higher wavelength) after being exposed to a light source of higher energy (lower wavelength). This type of emission may be fluorescence or phosphorescence; the difference being the former’s electron relaxation occurs at a rapid rate (~10 ns), due to the spin allowed transition from the excited state, like fluorescent invisible ink under black lights, while the latter occurs at a slower rate (> 1 ms), due to the spin forbidden transition, like glow-in-the-dark materials. Both fluorescence and phosphorescence are forms of photo-luminescence, which is one of many types of luminescence.28

Luminescence is the physical phenomenon that is observed when photons emit from a cool-bodied material. This is distinct from incandescence, where photons are emitted from a hot-bodied material. Other types of luminescence include the following: Chemiluminescence occurs when there is light emission from a chemical reaction, much like what happens when a glow stick is used and the hydrogen peroxide in the inner glass vial is mixed with the dye and diphenyl oxalate in the plastic tube.29 Electroluminescence occurs when light emission results from an electric current passing through a substance, much like neon lights. Radioluminescent light results from materials being bombarded with ionizing radiation. These materials include scintillators like sodium iodide and 2, 5-diphenyloxazole used for radiation detection. The previously mentioned photoluminescence involves materials absorbing photons and then observing photons emitting from them, and this is where the focus will be for the remainder of this work.
2.2 Photosensitization reactions and electron transitions

Physically, photodynamic therapy involves a combination of electron transition mechanisms to ultimately transfer a specific amount of energy and react with cancer cell components to promote cellular destruction. Figure 5 is a Jablonski diagram that depicts the processes of these electronic transitions to eventually generate radicals and singlet oxygen. Beginning from a source, energy in the form of light is exposed and then absorbed by the photosensitizer.

![Jablonski diagram of photosensitization reactions](image)

**Figure 5** Jablonski diagram of photosensitization reactions
Some electrons of the photosensitizer are then able to become excited, transitioning to an excited state ($S_n$), and then relaxed. During the relaxation process, those electrons can either go through a non-radiative internal conversion or relax quickly to the ground state ($S_0$), emitting a fluorescent photon as a result. The excited electron can also go through intersystem crossing to transition from the singlet state, where the electron pair’s spin is anti-parallel, to a triplet state, where the electron pair’s spin is parallel. From this triplet state, an electron can be transferred to its substrates and surroundings for a Type I reaction and create radicals. A Type II reaction occurs when the relaxing triplet state electron transfers energy to surrounding oxygen molecules to create singlet oxygen. With these capabilities in mind, nanoparticles and photosensitizers can be better designed to generate this form of oxygen and treat cancer for photodynamic therapy.\textsuperscript{30}

2.3 Protoporphyrin IX the photosensitizer

One of the key ingredients for photodynamic therapy, as previously mentioned, is the photosensitizer. It is the converter that can take photon energy and transfer to and generate singlet oxygen.\textsuperscript{31} The porphyrin photosensitizer, protoporphyrin IX, is well-known for its high quantum yield of 0.57, its ability to utilize a majority of the energy from incoming photons with minimal loss through undesired processes like non-radiative relaxation.\textsuperscript{32} Protoporphyrin IX can be found in nature as a product of 5-aminoluvelinic acid\textsuperscript{33} and also as an important precursor to heme \textsuperscript{34} and chlorophyll \textsuperscript{35}. The main difference is the metalation of the porphyrin core, which can give each porphyrin compound its unique properties. \textsuperscript{36}
Protoporphyrin IX comes from a family of porphyrins containing the parent core molecule Porphin. This core is made up of four pyrroles that are connected by four methane bridges as shown in Figure 6. Other compounds contain many similarities to the Porphin core including Chlorines and phthalocyanines, and although there are similarities, the differences between them lie with some slight extensions to the molecular

Figure 6 Protoporphyrin IX and macrocyclic cores
structure, less pyrrolic sections, and the number of pi-electrons. This macrocyclic system is highly aromatic and allows for a stronger absorption of light near the edge of UVA and purple’s band with a peak around 400nm and also weaker absorptions in the visible range with peaking around 500-700 nm, as can be seen in Figure 7.

![Absorption of Protoporphyrin IX](image)

Figure 7 Absorption spectra of Protoporphyrin IX with the Soret band peaking at 410 nm and Q-bands between 500 nm and 650 nm.

The spectroscopic characteristics of Protoporphyrin IX, along with the porphyrin family, are quite unique and piqued many interests, due to its absorption properties, both in application and in electronic theory. Spectroscopic information gives insight into the behaviors of electrons and to the energies that are involved during transitions. Usually, these would involve the valence and conduction bands of inorganic compounds and the
highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO) for organic compounds. With the given macrocyclic molecular structure and the absorption spectra, the work on the electronic theory for the porphyrin family required some unconventional polycyclic theories.

Now used as a strong basis for explaining porphyrins’ spectra, Gouterman proposed a four orbital model which suggests two HOMOs (HOMO-1, HOMO) and two LUMOs (LUMO, LUMO+1) are involved with two $\pi \to \pi^*$ transitions that are responsible for the two bands. The intense Soret band, with the transitions to the second excited state ($S_0 \to S_2$) giving a strong peak around 400 nm, is the result of the transition moments being additive while the Q band, with the transitions to the first excited state ($S_0 \to S_1$) giving a much weaker peak around 550 nm, results from the near cancellation of the transition moments. The metalation and the substituents on the ring are the factors responsible for the splitting and the intensities of the peaks of the Q bands. Figure 8 shows Gouterman’s four-orbital model and how changes in energy gaps influence transitions.
2.4 Protoporphyrin IX at present

Protoporphyrin IX is the only FDA approved photosensitizer from the group of porphyrin-related compounds. Aside from this, clinical and pharmacokinetic advantages such as high accumulation in tumor cells, fast elimination time of less than a day, and release of excess Protoporphyrin IX into the intestines have been found to be favorable qualities. Another advantage is that Protoporphyrin IX’s absorption, as seen in Figure 3, the Soret band is ten times more intense than the absorption at the Q bands. In terms of photodynamic therapy, this allows versatility in sensitizing Protoporphyrin IX by allowing...
the option of either penetrating energies or wavelengths. This photosensitizer is typically located in mitochondria, where it can be synthesized from succinyl coenzyme A and aminolevulinic acid, but accumulation of exogenous Protoporphyrin IX was also discovered. However, the location of the photosensitizer has been found to be a small factor as the inhibition of tumor growth and cancer cell killing effects were still capable. This removed the limit of having to focus solely on sub-cellular Protoporphyrin IX and revealed the possibility of administering it from an outside source.

Because of the penetrative ability of longer wavelengths, specific light sources are utilized for topical photodynamic therapy and diagnoses, where red light is used to penetrate tissues while still containing enough remaining energy, after scattering, for sensitization. For tissues near the surface of organs but inside the body, interstitial photodynamic therapy uses waveguides in the form of optical fibers to guide red light to treatment sites. With the ability to absorb light energy in a wide range from Ultraviolet to Infrared radiation, it is no surprise porphyrin-like compounds are an active part in photodynamic therapy.

Despite having unique spectroscopic features, the physical molecule itself has presented obstacles. Protoporphyrin IX contains four methyl groups, two vinyl groups, and two propionic acid groups as side chains of the porphyrin core, as seen in Figure 9,
and reactions by these chains with either itself, other Protoporphyrin IX molecules, or even the immediate molecular environment were found to affect its spectroscopic properties. Several typical observations were of the porphyrin core of one reacting with another’s pyrrole or propionic acid chain. The solvent is a major factor as well. Inorganic solvents have been used to easily dissolve Protoporphyrin IX and retaining its strong Soret peak. However, when dissolving in water, low concentration are used, and the pH strongly affects Protoporphyrin IX’s behavior. It remains as monomer in acidic solutions, aggregate at neutral pH, and dimer in basic solutions. To mitigate these interactions, other chemicals have been used to compete with the self-interactions and stacking in order to maintain
better solubility and absorption properties. This way, the problem of Protoporphyrin IX’s relation to its water solubility can slowly be resolved.

2.5 Instrumentation

2.5.1 Ultraviolet-Visible Spectroscopy

To measure absorption spectra, we use an Ultraviolet-Visible spectroscopy (UV-Vis). This is a tool to characterize some optics of samples by measuring the photon absorptions within the range of ultraviolet to visible radiation. This UV-Vis spectroscopy measures the electronic transitions from the ground state to the excited states. Not all materials can fluoresce, while all materials that can be seen has some amount of absorption. One of the benefits of using this technique is that it can indicate what sort of excited states a material can be excited to. This can be better seen when UV-Vis spectroscopy is paired with Fluorescence spectroscopy. In this work, we use a SHIMADZU UV-2450 spectrophotometer to measure the absorption of our nanoparticles and photosensitizers.

Typically, an UV-Vis spectrophotometer contains the following: an electromagnetic radiation source, a diffraction grating, a cell with either one or two cuvette holders, and a detector. Our model specifically had two cuvette holders; one for the reference sample and another for the working sample. Two sources are actually used in order to cover the Ultraviolet and the visible region of the electromagnetic spectrum. For UV region, a deuterium lamp is used, and for the visible region, a halogen or a tungsten lamp is used. During scans, the path of the light begins from the UV-Vis light source and then is separated into its component wavelengths by a diffraction grating and slits. Then, either the dispersing element or the exit slit can move. This way, only the radiation of a selected
wavelength leaves the monochromator through the exit. The light of a selected wavelength is then divided into two separate paths by a beam splitter, where one of the beams passes through the reference sample and the other passes through the working sample. The reference sample is supposed to contain only the solvent used for dispersing the sample or the solvent including anything else aside from the material in the working sample that is being observed. The two light beams are then collected and compared using a detector, which is usually either a photomultiplier tube, a photodiode, a photodiode array or a charge-coupled device (CCD).

The intensity the sample (I), the intensity of the reference (I₀) and the absorbance (A) for the given wavelength is related by the Beer-Lambert law given as

\[ A = \log \left( \frac{I_0}{I} \right) \, . \]

To obtain the final spectrum, the spectrophotometer calculates the absorbance for every wavelength that is accessible by its machinery, resulting in absorbance vs wavelength absorbance graph. For accurate measurement of absorption, it is required that the concentration of the sample is low to avoid any discrepancies due to light scattering. A schematic of the UV-Vis spectrophotometer is shown in Figure 10.
2.5.2 Photoluminescence Spectroscopy

Photoluminescence spectroscopy is a method to probe the electronic structure of materials by monitoring the photoluminescence, the emission of photons from materials, under photoexcitation. When a sample is excited by photons of a specific excitation wavelength, the sample may absorb some energy of the optical photons and then become photo-excited. During excitation, valence electrons from the electronic ground state shifts to a higher electronic state. When these excited electrons relax and return to the ground state, excess energy is released, which either may include the radiative process of the emission of light or some other non-radiative process such as vibronic relaxation, as mentioned previously. Due to the different relaxation paths, the light used for excitation and the light emitted from a sample will have a difference in energy. This energy difference between the excitation band’s maximum and the emission band’s maximum is called the...
Stokes shift. The PL excitation and the PL emission spectra of the organic scintillator 2, 5-diphenyloxazole are shown in Figure 11. The Stokes shift can be seen to be 35 nm.

**PL: PPO**

![Photoluminescence spectra of PPO](image)

Figure 11 Photoluminescence spectra of PPO

Both the excitation and the emission spectrum can be measured, which can be easier with an accompanying computer program like Panorama. The excitation spectrum is a distribution of wavelengths recorded when keeping an emission wavelength constant, while the emission spectrum is the distribution of wavelengths detected when keeping an excitation wavelength constant. As a result, the emission spectrum is measured with a photoluminescence emission format, PL, and the excitation spectrum is measured with a photoluminescence excitation format, PLE. In the PL format, a specific wavelength is selected to excite the sample and the emission is monitored within a specified emission range. In the PLE case, an emission wavelength is selected to measure the wavelengths of the optical photons that stimulate the emission. Some factors to note are artifact signals.
These can originate from either the spectrofluorophotometer or the sample chamber and may give extreme and false signals not intended from the sample.

For this work, we used a SHIMADZU RF-5301 PC Spectrofluorophotometer to measure the PL and PLE of the nanoparticles, the photosensitizers, the scintillator, and other solutions. A diagram of a spectrofluorometer is shown in Figure 12. In general, a spectrofluorometer consists of a light source, an excitation monochromator, a sample chamber, an emission monochromator, and a detector. A xenon lamp was used as a light source to produce photons in the 220-990 nm range in Shimadzu RF-5301 PC. The path begins at the source as it sends out light to be used as the excitation wavelength towards a sample. Before it reaches the sample, the light passes through the excitation monochromator, which transmits a selective wavelength for the excitation spectrum measurement while blocking other wavelengths. The light from the excitation monochromator passes through the sample contained in the sample compartment and excites the sample. After excitation, the sample emits light at an emission wavelength usually longer than the excitation wavelength. The emitted light passes through the emission monochromator positioned at a right angle to the excitation light. The emission monochromator minimizes scattered light and screens the emission light before it reaches the detector. The detector measures the emitted light and displays the fluorescence spectrum given by the sample.61
2.5.3 X-ray Excited Optical Luminescence

X-ray excited optical luminescence (XEOL) is an optical characterization technique where the photoemission from the sample is measured under X-ray irradiation. In this work, an RX-650 Faxitron X-ray cabinet system is used for X-ray irradiation. The schematic of Faxitron Standard X-ray tube is shown in Figure 13. The tube consists of an X-ray tube with an X-ray generator to supply the X-rays beams. The current is fixed at 3 mA, and the acceleration voltage can be tuned with a maximum value of 130 kV\textsubscript{p}. The X-ray generator supplies the current which is then heats up a filament to produce electrons. The electrons
are then accelerated through the high voltage and bombarded onto a tungsten target to produce both bremsstrahlung and characteristic and bremsstrahlung. The angle of the anode is 20° and the focal spot size is 0.5 mm. The X-ray tube is equipped with a 0.76 mm beryllium window to allow a full spectrum of soft X-ray output.

Figure 13 Schematic outline of the X-ray tube

The X-rays will excite the sample and the sample will exhibit luminescence upon de-excitation and the luminescence spectra was monitored using a QE65000 spectrometer from Ocean Optics Inc., which was coupled to the X-ray cabinet. The spectrometer separates the light into individual wavelength components using a grating monochromator. Then a charge-coupled device records the intensities of the individual components of wavelength. Typically, X-ray tube is operated with 3 mA current and 90 kVp voltage. The
output from a typical XEOL experiment is the intensity distribution in counts per seconds of the scintillation light versus wavelength as shown in Figure 14.

![Figure 14 XEOL spectra of PPO with a typical peak around 400 nm.](image)

**2.5 Thesis**

Protoporphyrin IX’s advantages over other similar structured photosensitizers makes it a priority subject for doing research. Its existence in cellular processes is evident for biocompatibility, and FDA approval has allowed it to be used in clinical treatments like topical and interstitial photodynamic therapy. In these treatments and research, emphasis of the use of red light is based on the characteristic absorption spectra, which is attributed to the unique aromatic macrocyclic molecular structure of the porphyrin core. However, the
absorption of Protoporphyrin IX extends to the UV region, displaying approximately ten times more intense in that region than that of the red region. In actuality, these characteristics can be seen mainly in organic solvents while barely seen in aqueous solvents and in low concentrations. This hinders applications for non-invasive deep cancer treatments. In order to apply Protoporphyrin IX for deep cancer PDT, its properties in aqueous solutions must be improved, they must be comparable to those in organic solvents, and then its improvement should allow it to be excitable by X-ray.

In this dissertation, we investigate the improvement and application of the organic photosensitizer, Protoporphyrin IX (PPIX), with latex polymer particles synthesized from styrene and 4-vinylpyridine monomers for deep tissue photodynamic therapy. Chapter 3 will present the improvement of PPIX’s aqueous properties by applying the polymeric nanoparticle for photodynamic therapy. Chapter 4 will present a further investigation of the effects of the particle synthesis with PPIX’s spectroscopic properties in aqueous solutions. Chapter 5 will present the addition of the organic scintillator molecule 2, 5-diphenyloxazole to the latex polymer particle and the application of Forster resonance energy transfer which will allow X-ray energy to activate the particle system for photodynamic action. Chapter 6 will summarize and conclude with future approaches and alternative perspectives for the project.
Chapter 3 Improvement of Protoporphyrin IX Properties in Aqueous Solutions with Latex Carriers for photodynamic therapy

3.1 Introduction

Photodynamic therapy (PDT) is one type of cancer treatment that has fewer side effects than other cancer modalities. PDT requires three ingredients in order to for its effect to work: a photosensitizer, a light source, and oxygen. The light source excites the photosensitizer from its ground state to the excited state. Afterwards, from the excited state, two reactions can occur: the first reaction forms radicals from the surrounding substrates, while the second reaction produces singlet oxygen from the ground state triplet oxygen. The efficacy of PDT depends on the efficacy of the light excitation source, the available oxygen, and the photosensitizer. PPIX has been used for clinical research, and its ability to accumulate in tumor cells makes it a preferable drug for cancer treatment. However, due to its amphiphilic nature, PPIX is prone to aggregate in aqueous environments either via π-π stacking or intramolecular interactions between the hydrophilic propionic acid's -COOH group and the hydrophobic porphyrin core. Some methods to avoid PPIX aggregation have been found to include modifying the –COOH with APTES and Folic acid and also allowing PPIX to be carried by nanoparticles. In addition to aiding PPIX avoid molecular aggregation, nanoparticles can allow further modifications to overcome limitations of PDT including increasing efficiency of cellular drug uptake by drug encapsulation and enabling deep tissue PDT with self-lighting nanoparticles. In this study, to improve PPIX's properties, including water solubility, dispersibility, stability, fluorescence, singlet oxygen generation and cellular uptake, we attach PPIX to poly(styrene-4-vinylpyridine) (PS4VP)nanoparticles via adsorption by hydrogen bonds.
This nanoparticle is expected to carry PPIX and keep PPIX from aggregating, thereby, increasing its solubility and dispersibility in aqueous media and allowing improved fluorescence, increasing singlet oxygen generation rate, and improving cellular uptake.

### 3.2 Materials and Experimental

Protoporphyrin IX ≥ 95%, 4-vinylpyridine (4VP), benzoyl peroxide (BP), styrene (Sty), sodium dodecyl sulfate (SDS), trypsin, penicillin were purchased from Sigma Aldrich. PC-3 (ATCC, passage #5) were cultured in RPMI (ATCC) supplemented with fetal bovine serum (FBS) and penicillin streptomycin (PS). 3- (4, 4- Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT) reagent and phosphate buffer saline (PBS) were purchased from Trevigen, and FBS and PS was purchased from Atlanta Biologicals. Sytox Green and DAPI were purchased from Thermo Fisher Scientific. All solvents and organic chemicals were of reagent grade.

#### 3.2.1 Synthesis of Poly (Styrene-co-4-vinylpyridine) nanoparticles

The procedure for synthesizing PS4VP was adapted and modified from several literatures. In short, for the synthesis, a bottom-up, oil-in-water heated emulsion polymerization technique was used. The aqueous phase was made by dissolving the surfactant Sodium Dodecyl Sulfate (SDS) in DI water. The oil phase consisted of 4VP and Sty monomers with dissolved BP as the radical initiator. Once the aqueous solution is stirring vigorously and heated to 90 °C, add the oil phase drop wise to the aqueous solution. Allow the synthesis to remain stirring and heating at 90 °C for 12 hours. At a specific temperature, in this case 90 °C, radical initiators can auto ignite and initialize the
polymerization. Initially, the emulsion will look opaque, and as the polymerization continues, the solution will become transparent containing the colloidal particles. Sizes were found to be less than 200 nm in diameter.

3.2.2 Preparation of Protoporphyrin IX attached Poly (Styrene-co-4-vinylpyridine) nanoparticle (PPIX-PS4VP)

To attach PPIX and complete the nanoparticle assembly, PPIX was added to the solution of PS4VP and then sonicated in a bath sonicator.

3.2.3 Preparation of PPIX in an SDS aqueous solution

PPIX was able to be delivered to cells by nanoscopic micelles, and singlet oxygen was also detected. A comparison with SDS solution may help show any increasing effects of PPIX between the solution with and without the PS4VP particles. An equal amount of PPIX was simply dissolved in an SDS aqueous solution for this comparison.

3.2.4 UV-vis absorption and Fluorescence Spectroscopy

A Shimadzu RF-5301PC spectrofluorophotometer was used to record fluorescent emissions of PPIX suspended in water, in aqueous SDS, and with PS4VP carriers at concentrations starting at 100 μg/mL. And an UVvis spectrophotometer (UV-2450) was used to record the absorbance spectra of PPIX in the three conditions.
3.2.5 *Solubility and stability*

Improvement in solubility and dispersibility of PPIX with a carrier is compared to PPIX in water and PPIX in aqueous SDS, and the three samples were allowed to age and allowed for precipitation by gravity.

3.2.6 *Singlet oxygen generation*

To determine singlet oxygen generation, we used an indirect singlet oxygen detection method for aqueous solutions involving p-nitrosodimethylaniline (RNO) and imidazole (Im). In short, a 3 mL aqueous solution containing the singlet oxygen generator, 50 μM of RNO and 8 mM of Im was used. The detection for absorption intensity of RNO near 440 nm was monitored by a UV-vis spectrophotometer after time intervals of ultraviolet exposure with simultaneous addition of oxygen.

3.2.7 *Cell uptake studies*

PC3 prostate cancer cells were used to observe and compare cell uptake of PPIX in DI, PPIX in DI-SDS, and PPIX-PS4VP in vitro. The cells were cultured in F-12K medium supplemented with 10% fetal bovine serum and 10% penicillin at 37°C and 5% CO₂. After allowing PC3 to be incubated with DAPI and with equal concentrations of PPIX from the three samples and after washing, images were viewed and taken with a fluorescent microscope to observe DAPI and PPIX fluorescence.
3.2.8 PDT Simulation with fluorescence imaging

PDT was simulated by irradiating 403 nm light onto a circular region of PC3 cells after having taken up PS4VP-PPIX to simulate and kill cells. Afterwards, membrane selective SYTOX green was used to bind only to the nuclear material of dead cells in order to show the cells affected by the UV radiation. Finally, the fluorescent microscope was used to observe the red fluorescence of PPIX and green fluorescence from SYTOX.

3.2.9 Cell viability of PDT-simulation with MTT

PC3 prostate cancer cells were used to test for any increased PDT capabilities of PS4VP-PPIX nanoparticles. To do this, two 96-well plates were concurrently seeded with 3000 cells per well and allowed to grow for one day. During the following day, after washing with PBS, PPIX-DI, PPIX-SDS, and PS4VP-PPIX were introduced to the wells at different concentrations of 25 μg/mL, 2.5 μg/mL, and 0.25 μg/mL. Both would be covered with aluminum foil to avoid unwanted UV exposure from the incubator. Before MTT reagent was introduced to both plates, one was used as a control with no UV exposure while the other was exposed to UV light for 1 minute to simulate the administering of light after the introduction of the photosensitizers.
3.3 Results and discussion

3.3.1 Solubility and stability of PS4VP-PPIX

After making equal concentrations of PPIX in water, SDS solution, and with PS4VP particles, the three samples were subjected to UV exposure and also allowed to age and precipitate. Figure 15 shows and describes the three samples in room light and under UV after allowing for precipitation. Under UV exposure, PPIX’s red fluorescence can be seen from the SDS solution and the PS4VP solution but not from the water solution. In the surfactant solution, SDS micelles are formed which help solubilize PPIX.
into solution. In the solution of PS4VP, similar to other macromolecular formations, a carboxyl group from PPIX adsorbs to the surface of the particle by hydrogen bonding to the nitrogen group of the 4-vinylpyridine, as shown in Figure 16. This helps prevent PPIX from aggregating by inter and intra molecular bonding and creating dimers and other aggregates. Keeping the carboxyl group of PPIX from interacting with its porphyrin core is an effective strategy which has also been applied by Homayoni et al by modifying the propionic acid chain directly. The approach to use PS4VP’s surface for hydrogen bonding may permit further modification with compounds capable of bonding in the same

Figure 16 Schematic of a PS4VP-PPIX particle with H-bond occurring between the carboxylic acid and the particle surface.
After letting the three PPIX solutions to stand and age for several days, it can be seen that the aqueous and SDS solutions remain unstable compared to the solution with PS4VP. PPIX precipitate can be seen settled with no fluorescence in Samples 1 and 2, and in Figure 15, a darker transparent supernatant with little to no precipitation can be seen in Sample 3. The increased solubility of PPIX with PS4VP will increase PPIX’s capability to absorb light while in aqueous solutions and allow applications involving an aqueous environment to benefit from PPIX’s absorption and fluorescence properties.
Figure 18 PL spectra of PPIX in DI water

Figure 19 PL spectra of PPIX in DI-SDS
Figure 20 PL spectra of PS4VP-PPIX

Figure 21 Photoluminescent intensity of different concentrations of PPIX

Increase of PPIX fluorescence at 639 nm by concentration and suspension medium excited with 400 nm
3.3.2 Spectroscopic properties of PPIX in DI, PPIX in DI-SDS, and PS4VP-PPIX

The characteristics of the absorption spectrum of PPIX are the typical Soret band and the Q-bands. Figure 17 shows the absorption spectra of equal amounts of PPIX dissolved in DI, DI-SDS, and with PS4VP. The Soret peak stands out in solutions with SDS and with PS4VP, while it does not in DI without SDS or PS4VP. This shows that PPIX has greater absorbance capacity when in solution with SDS and PS4VP, and that the Soret peak, marked between the dashed lines in Figure 17, depends on the solution environment. In this case, the addition of SDS and PS4VP is the factor that improves PPIX’s absorbance at the Soret peak. Also, it can be seen that fluorescence under UV exposure depends on similar factors.

With the three samples containing equal concentrations of PPIX, in Figure 8, PPIX showed an increase in fluorescence at 639 nm when excited by 400 nm while attached to PS4VP, compared to the surfactant solution with the aqueous solution showing the least fluorescence. There seems to be a particular concentration of 50 μg/mL of PPIX in these solutions where PPIX can emit the strongest fluorescence. In addition, Figure 8 also shows that being attached to the particle greatly improves PPIX fluorescence, which is a sign of higher water solubility and higher absorption of light which can lead to a higher increase of PPIX’s singlet oxygen generation rate.
3.3.3 Singlet Oxygen detection

Having PPIX attached to PS4VP allows PPIX to have better fluorescence which can be a sign of a better rate of singlet oxygen generation. To see how the factors of SDS and PS4VP affect the rate of singlet oxygen generation, the RNO-Im was used with UV light exciting the PPIX solutions, initiating the process needed to generate singlet oxygen. Singlet oxygen would react with Imidazole producing and trans-annular peroxide (TAP), which would then react and bleach RNO, causing RNO absorption to decrease. RNO's absorption is monitored around 438-440 nm, and can be seen decreasing after the simultaneous addition of oxygen with exposure to UV light, as can be seen in Figure 22.
Little to no decrease in RNO absorbance was seen before 10 seconds of reaction time; however, as the amount of time for the reaction to occur increase, the absorbance of RNO with PS4VP-PPIX continued to decrease more than the absorbance of RNO with PPIX-SDS and PPIX-DI, while the absorbance of RNO with PPIX-SDS remained less than the RNO with PPIX-DI. This continuous decrease of RNO with PS4VP-PPIX indicates an increased rate of singlet oxygen generation, which follows the particles’ better absorbance and fluorescence.

There is a scarce amount of methods that try to quantify generated singlet oxygen ranging from directly detecting the 1270 nm phosphorescence emission from it to spectroscopic yield comparisons, which depend on various rates that depending on the generating environment. By correlating RNO’s concentration with its absorbance, shown in Figure 23, and assuming one singlet oxygen molecule affects one Imidazole molecule to bleach one molecule of RNO, a calculation results in an estimated 1.44 uM of $^1O_2$ molecules generated.

![Correlation: Absorbance vs Concentration of RNO in DI](image)

Figure 23 Plot of RNO concentration correlation
Figure 24 Fluorescence shown of with DAPI in PC3, (a) PPIX-DI, (b) PPIX-SDS, (c) PS4VP-PPIX. Uptake was allowed to occur within 24 hrs. DAPI was used to visualize cells with blue fluorescence while red fluorescence can be seen from the 3 samples.
3.3.4 Cell uptake experiment

It is expected that, with the attachment of PPIX to PS4VP, more PPIX can be taken up in cancer cells. After the accumulation of PPIX from the three solutions, red fluorescence from PPIX can be seen under 403nm excitation in Figure 24. In addition, there is more of PPIX’s red fluorescence with DAPI’s blue fluorescence from the cells incubated with PS4VP-PPIX compared to the aqueous and surfactant solutions. PS4VP-PPIX can be seen dispersed within cells along with DAPI, showing an increase in uptake of PPIX. Increased uptake is greatly desired in hopes that it leads to a greater efficacy. PPIX initially had low cell uptake, but with PS4VP, more of the particles can be taken up, allowing a better chance at using its red fluorescence for imaging and a better chance at generating more singlet oxygen for PDT.

Figure 25 Method to image PDT treatment. After administering the PS4VP-PPIX, irradiate and area for 1 minute, and then add and allow SYTOX green to be taken up by cells and visualize the fluorescence of irradiated and non-irradiated region.
Figure 26 Bright field of PC3 cells after treatment. The dashed line separates the areas irradiated by 403 nm light (right) and the non-irradiated area (left).

Figure 27 Fluorescence of SYTOX green in treated PC3 cells. The dashed line separates the areas irradiated by 403 nm light (right) and the non-irradiated area (left).
Figure 28 PPIX fluorescence in PC3. The dashed line separates the areas irradiated by 403 nm light (right) and the non-irradiated area (left).

Figure 29 Merged image of SYTOX green and PPIX. The dashed line separates the areas irradiated by 403 nm light (right) and the non-irradiated area (left).
3.3.5 PDT simulation experiment with fluorescence imaging

After irradiating with 403 nm in the small circular region of a 6-well multiplate, as shown in Figure 25, cell morphology could be seen in Figure 26 to have changed tremendously when compared to an area outside of the irradiated region. The irradiated area is shown as the filled purple circular region. Furthermore, the addition of the SYTOX green stain, with fluorescence shown in Figure 27, indicated that, of the cells that were incubated with PS4VP-PPIX, as shown in Figure 28, only the region that was irradiated with UV contained dead cells. The dead cells can be seen in the upper right of the images in Figures 26. The area outside of the irradiated region shows good dark toxicity, when PS4VP-PPIX would not activate until exposed to light. The green fluorescence from SYTOX green indicates cells that have died, and under fluorescence microscopy in Figure 29, the irradiated region is the only area that contains dead cells. With the changed cell morphology and the green emission from the selective SYTOX green under the irradiated area, cells were clearly killed. And with PS4VP-PPIX fluorescence and a lack of SYTOX green fluorescence in cells outside of the irradiated area, this signifies that PDT occurred and that PS4VP-PPIX is safe for untreated cells and is capable for PDT treatment.

3.3.6 MTT cell viability of PDT simulation

A comparison of the PDT effect of the three samples was conducted with two 96-well plates. Figure 30 shows cell viability after being treated with UV for 1 minute. Control groups that were incubated with the three samples and not irradiated by UV maintained above 80% cell viability. When UV exposure was applied, cell viability of cells incubated with PPIX-DI decreased slightly but still about 80%, signifying little PDT action. Cells
incubated with PPIX-SDS and PS4VP-PPIX showed 31% and 6% cell viability, respectively, signifying PDT action occurring from both solutions of PPIX. The decrease of cell viability signifies the increased capability of PDT with PPIX, especially when attached to PS4VP.
3.4 Conclusion

The amphiphilic photosensitizer PPIX aggregates into supramolecular assemblies in water leading to poor water solubility and low luminescence. This limits the range of its usage including for medical purposes and other applications involving aqueous environment. In this paper, we reported the use of latex beads with nitrogen at the surface to help carry and improve PPIX by having the carboxylic acid groups bond to the carrier, and keep it from reacting with its hydrophobic core. In doing so, this helps prevent aggregation while increasing PPIX luminescence, dispersibility in water, singlet oxygen production rate. Adsorption to the surface may allow further applications, as other compounds with –COOH groups can take advantage of the PS4VP’s surface. We also observed better cellular uptake and photodynamic capabilities. Increased cell uptake allows a better use of the intense red fluorescence and can help improve imaging applications. This can be used in conjunction with the increased singlet oxygen generation to allow both imaging and treatment to occur simultaneously. With these improvements and further desirable exploration, this PS4VP-PPIX carrier and photosensitizer can be used for enhanced PDT with PPIX and help to improve other photosensitizers’ accessibility for photodynamic therapy.
4.1 Introduction

Photodynamic therapy (PDT), a cancer treatment along with surgery, chemotherapy, radiation therapy and others, contains the benefits of being a minimally-invasive form of treatment which uses non-toxic photosensitive chemicals that can be activated by selective wavelengths of light from the broad electromagnetic spectrum. This then leads to two types of reactions that can generate cancer killing agents from substrates and oxygen\(^1\). One specific porphyrin photosensitizer, called Protoporphyrin IX (PPIX), is used in PDT and is still a subject of many interests ranging from molecular and nanoparticle research, involving porphyrin particle fabrications and molecular property enhancements, to clinical research, concerning with optimizing treatment methods and conditions for patients\(^2\). A major drawback of using photosensitizers like PPIX is water solubility; the low solubility of this organic molecule in water is typically results in hindered properties including weak light absorption and emission which in turns gives low PDT prospects\(^3\).

To get around these hurdles, some research groups would work with PPIX in organic solutions instead of aqueous solutions while others would use low concentrations of PPIX in aqueous solutions\(^4\). Other groups have developed nanoparticles and other modifications to improve PPIX’s water solubility and its properties in aqueous solutions\(^4,5\). Applications have shown the validation of the newly enhanced PPIX particles, yet few studies investigate further into the effects and consequences on PPIX such as photobleaching, its fluorescent properties, and singlet oxygen generation after having been modified and improved. In this paper, we synthesize poly (styrene-co-4-vinylpyridine)
nanoparticles (PS4VP) using different amounts of monomers to observe the effects of ratio of initial monomers on PPIX, its photo-properties after bonding to PS4VP, and its capabilities of generating oxygen in aqueous solutions.

4.2 Materials and Experimental

4.2.1 Materials

Chemicals from Sigma Aldrich included the following: Styrene (Sty) and 4-vinylpyridine (4VP) were used as monomers. Benzoyl peroxide (BP) was used as the crosslinking radical initiator. Sodium dodecyl sulfate (SDS) was used as the anionic surfactant and Protoporphyrin IX (PpIX) was used as the porphyrin photosensitizer. Singlet Oxygen Sensor Green (SOSG) was purchased from Thermo Fisher. The DI water was used after having gone through a double purification system. All solvents and organic chemicals were of reagent grade.

4.2.2 Synthesis of Poly (Styrene-4-Vinylpyridine) particles

For the synthesis, a bottom-up emulsion polymerization was used. This can be seen in Figure 31. The monomer solution contained styrene and 4-vinylpyridine, while the aqueous solution contained sodium dodecyl sulfate as the surfactant. The aqueous solution was stirred vigorously and heated to 90° C in a round bottom flask, and as soon as it reached 90° C, the oil solution was prepped by adding in to it the radical initiator benzoyl peroxide. After the initiator dissolved, the monomer solution was then added slowly and dropwise into the aqueous phase that was kept stirring and heating. The synthesis was left for 6 hours for the polymerization to finish. Afterwards, once the nanoparticle
solution was allowed to cool down to room temperature, a portion was dialyzed while the remainder was used to complete the final particle.

4.2.3 Varying 4-vinylpyridine in synthesis

The initial monomer content was varied to observe effects of attachment with PPIX. To this end, the mass of the total initial monomer solution was maintained at 1g. 4VP and styrene’s mass was varied to show increasing 4VP with decreasing styrene. All other conditions of the synthesis were kept constant, including the mass of initiator used. During the syntheses, the initial monomer solution darkened with increasing 4VP, and after adding them into the aqueous phase, the syntheses solutions were seen to change from opaque to transparent.

4.2.4 Attachment of PPIX to nanoparticle

To attach PPIX to the surface of PS4VP, the particle solution was used after it had cooled down to room temperature. Equal quantities of PPIX was dissolved into the solution with the help of a bath sonicator. PPIX dissolution can be observed with an accompanying red fluorescence when exposed under UV light. For the varied nanoparticle solutions, a constant quantity of PPIX was added to each. After sonication for 1 hour, the solutions were left for any unattached PPIX to precipitate, and the supernatant were collected for analysis. The final solutions remained stable, transparent, and red in normal room lighting.
- Synthesis: Bottom-up Oil-in-water emulsion copolymerization
4.2.5 Spectroscopy

A Shimadzu UV-2450 UV-vis spectrophotometer was used to ascertain the absorption spectra of PPIX and PS4VP after synthesis. Two cuvette holders were accessible so that standard and sample solutions could be scan simultaneously without having to risk affecting the standard’s baseline absorption. A Shimadzu RF-5301PC spectrofluorophotometer was used to obtain the excitation and emission spectra of the particles. Solutions samples were scanned as synthesized and diluted to determine optimal fluorescence and avoid self-quenching and saturated absorption. Dried powder samples were also scanned to determine emission characteristics of PS4VP-PPIX.

4.2.6 Sensing Singlet Oxygen Sensing

The Singlet Oxygen Sensor Green reagent was used as the highly selective sensor for singlet oxygen, so as to not risk inadvertently detect hydroxyl radicals or superoxide anions. 10 uM of SOSG was used as the working solution with PS4VP-PPIX samples to compare rates of singlet oxygen generation. After initial scans, solutions were subjected to UV exposure to activate PPIX, generate singlet oxygen, and then react with SOSG, increasing SOSG’s fluorescence, which could then be detectable.

4.3 Results and Discussion

4.3.1 Synthesis with 4VP variation
During synthesis, we controlled the initial total masses of the monomer solution and radical initiator as 1 g and 50 mg, respectively, and then varied the separate masses of the styrene and 4-vinylpyridine monomer as shown in Table 1. Some groups have used different combinations of styrene and 4-vinylpyridine to synthesize nanocomposites and nano-ellipsoids, with some emphasis into the variation of the two monomers, for antifungal, antibacterial, and scaffold applications. In our work, we hypothesized that, by increasing the quantity of 4VP, the locations for hydrogen bonding between the nitrogen of 4VP and COOH- groups of PPIX will also increase and, ideally, PPIX would be distributed differently along the particle surfaces of as depicted schematically in Figure 32. The distribution of PPIX along the surface would likely be increased causing increased turbidity. This was visibly seen in Figure 33 as the color of the solutions initially looks transparent with a lighter red hue and then becomes less transparent with a darker red hue as the initial quantity of 4VP monomer increased.

<table>
<thead>
<tr>
<th>Sty:4VP mol ratio</th>
<th>Styrene mmol</th>
<th>4VP mmol</th>
<th>Sum of oil mmol</th>
<th>Initiator mass (g)</th>
<th>DI volume (mL)</th>
<th>SDS mass (g)</th>
<th>Temp (°C)</th>
<th>Heat time (h)</th>
<th>PPIX/soln mass conc (_mg/4mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>5:3</td>
<td>6.00</td>
<td>3.57</td>
<td>9.5</td>
<td>0.050</td>
<td>50</td>
<td>0.5</td>
<td>90</td>
<td>5.5</td>
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<tr>
<td>2.</td>
<td>4:4</td>
<td>4.80</td>
<td>4.76</td>
<td>9.5</td>
<td>0.050</td>
<td>50</td>
<td>0.5</td>
<td>90</td>
<td>5.5</td>
</tr>
<tr>
<td>3.</td>
<td>3:5</td>
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<td>5.94</td>
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<td>90</td>
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<tr>
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<td>50</td>
<td>0.5</td>
<td>90</td>
<td>5.5</td>
</tr>
</tbody>
</table>

Table 1 Measurement quantities for varying 4VP for synthesis
4.3.2 Spectroscopic data

The photoluminescence spectra of the PS4VP particles, shown in Figure 34, showed slight shifts in the excitation spectra but indistinguishable shifts of the emission peaks, indicating the relationship between photoluminescence and styrene and 4VP initial quantities are negligible.
Figure 34 PL spectra of as synthesized PS4VP showing negligible fluorescence dependence on initial PS4VP content

After the completion of each samples with PPIX, the absorption spectra of the PS4VP-PPIX particle solutions showed the characteristic Soret peak and Q peaks that can be seen for many porphyrin-based compounds. Having this shape at the Soret peak is an indication that there are little to no PPIX dimers or aggregates in the solution, and it also signifies that PPIX when bonded to PS4VP can absorb light better as opposed to PPIX aggregates which have lower capabilities to absorb light and fluoresce in water. This was also shown by Bui et al when trying to apply this particle for enhancing cancer therapy.
PS4VP-PPIX samples were treated with UV while aeration to observe effects on the absorption spectra. As shown in Figures 35 and 36, there may be signs of photobleaching. Comparing the change of the peak intensities, the Soret peak at 410 nm decreased while the typical Q peaks at 507 nm, 542 nm, 575 nm, and 630 nm showed extremely little change. Another peak that was initially not present at 670 nm emerged and increased over time with UV exposure, shown as the dashed green line in Figure 37. The development of this peak is similar to the ones that were found when treating PPIX in DMF, where they found photo-oxidation of PPIX to occur and a photoproduct to be present.\textsuperscript{10}

Figure 35 Absorption spectra of treated PS4VP-PPIX showing typical porphyrin peaks in the Soret band and Q-bands.
Figure 36 Change of peak intensities after 30 s UV exposure. All peaks shown include the Soret peak, the peaks at the Q-band, and the photoproduct’s peak.

Figure 37 Expanded view of changes of absorption intensities after UV treatment shows a dramatic increase of absorption at 670 nm compared the typical Q-bands.
The photoluminescence spectra of PS4VP-PPIX in Figure 38 show the emissions that were present in the sample after UV exposure with aeration. The 641 nm emission is red-shifted from the 633 nm emission that is normally seen from PPIX. The emission at 704 nm had been also slightly red-shifted from 701 nm, which is also seen in free PPIX. These occurrences are most likely due to PPIX bonding to PS4VP. The spectra reveal a decrease of both 641 nm and 704 nm, signifying a photo-bleaching effect of PPIX. However, there was also an increase in the 680 nm emission signifying a production in effect. These changes over time can be seen in Figure 39. This emission is most like resulting from the red-shifting of an emission that would be normally seen around 670 nm.

Figure 38 PL spectra of PS4VP-PPIX after 30s of UV and aerations with significant peaks at 641 nm, 681 nm and 707 nm.
Figure 39 PL changes after UV exposure and aeration show decreasing emissions from 641 nm and 707 nm but then increasing from 681 nm with continual UV exposure.

The decreasing of PPIX emissions and Soret peak with the development of an absorption at 670 nm and new emission at 680 nm leads to the conclusion that a photoproduct was developed during the UV irradiation with aeration. This product can be attributed to PPIX being photo-oxidized into the photoproducts including photoprotoporphyrin\textsuperscript{11}. These were found to be due to a cyclo-addition of singlet oxygen either to the diene unit to form the hydroxyaldehydes photo-protoporphyrin or to the vinyl group to form formyl porphyrins, and despite having high quantum yields; these are more stable towards photo-oxidation than PPIX\textsuperscript{12-15}. Being attached to the surface of PS4VP by the carboxylic acid groups of the propionic chains, the vinyl groups still remain to reactions. Compared to previous studies on PPIX reactions and photobleaching in organic solvents,
these spectra were obtained in aqueous solutions. This applicability in aqueous solution with the increase of stability due to the photo-modification process during photo-oxidation may allow more studies of applications involving water and aqueous environment like water treatment and interstitial PDT\textsuperscript{16} by shifting from PPIX’s photo-bleaching as the outcome to its photoproducts’ photo-bleaching in order to maximize treatment and consider the whole true photo-bleaching process.

4.3.3 Singlet Oxygen Generation Detection

SOSG has been used as a fluorescent probe to selectively detect singlet oxygen and to avoid detecting other reactive oxygen species such as hydroxyl radicals and superoxide anions. As more singlet oxygen becomes generated, SOSG fluorescence increases\textsuperscript{17-19}. This was simulated by the aeration as the samples were irradiated with UV. Here, it is also used to verify the capabilities of the varied PS4VP-PPIX to generate singlet oxygen with increasing rate relative to increase amounts of PPIX. Figure 40 plots the fluorescence intensity of SOSG treated along with PS4VP-PPIX samples and monitored at 525 nm, and Figure 41 shows how SOSG’s emission remains increasing as PPIX.
Figure 40 Singlet Oxygen Detection by SOSG Fluorescence shows that singlet oxygen from PPIX seems to be generated when more 4VP is present in PS4VP particles.

Figure 41 SOSG emission and PPIX emission trend show the emission of PPIX at 681 nm continues to increase as SOSG increases, signifying singlet oxygen generation still occurs as PPP is generated.
Equal aliquots of each sample were mixed with equal concentrations of SOSG in order to control the initial emission of SOSG. With the increasing SOSG fluorescence across many runs, it can be seen that all samples produced singlet oxygen during the 30 seconds exposure to UV with aeration. Contrastingly, PS4VP-PPIX samples synthesized with more initial 4VP showed greater rates in singlet oxygen generation. This is most likely indicative of larger quantities of PPIX in the aliquots used. These generations of singlet oxygen with the production of stable porphyrin photoproducts from our particles now in aqueous solutions are in agreement with studies of PPIX in organic solvents, which also show stable photoproducts and maintenance of singlet oxygen generation from these products²⁰.

4.3.4 Temperature dependent Photoluminescence

PS4VP-PPIX’s fluorescence property was also observed against temperature and can be seen in Figure 9. Each spectrum was collected after samples were subject to the temperature shown. From 27 C to 227 C, the emission peaks of Protoporphyrin IX and Photoprotoporphyrin can be seen, with the lowest detectable emissions at 227 C. At 27 C, 77 C, and 127 C, photoprotoporphyrin’s emission were stronger than PPIX’s, and both decreased as temperature increased. As shown in Figures 42-50, at 177 C and 227 C, photoprotoporphyrin’s emission became less dominant than PPIX’s, this is could be due to the loss of the hydroxyl groups that were added to PPIX by singlet oxygen. It makes sense that as those were lost, the porphyrin structured returned to PPIX, which then corresponds to the increase in PPIX emission. Although the experiment omitted a reading at a body temperature, it can be safe to suggest the spectrum would retain the peaks as seen from the ones at 27C and 77C. After 227 C, no emissions related to the porphyrins were
observed. This is likely due to the compound becoming denatured and therefore losing its luminescence capabilities.

Figure 42 PL spectra after 27°C temperature treatment

Figure 43 PL spectra after 77°C temperature treatment
Figure 44 PL spectra after 127°C temperature treatment

Figure 45 PL spectra after 177°C temperature treatment
Figure 46 PL spectra after 227°C temperature treatment

Figure 47 PL spectra after 277°C temperature treatment
Figure 48 PL spectra after 327°C temperature treatment

Figure 49 PL spectra after 377°C temperature treatment
4.4 Conclusion

We report the synthesis of variable porphyrinated latex particles. By adjusting initial 4VP monomer quantities, different amounts of PPIX can bond to the surface. This allows loading of PPIX and results in increasing amounts of PPIX as initial 4VP is increased. These variable PS4VP-PPIX particles showed preservation of PPIX properties including photomodification into its more stable photoproducts, which created another fluorescence emission peak at 710 nm, which may be beneficial for imaging. Generation of singlet oxygen was also found to increase as 4VP and PPIX was increased. Future works may be expanded to include other chemicals that can take advantage of the variable surface of PS4VP. As they were synthesized in aqueous solutions and kept PPIX’s properties usually
found when dissolved in organic solvents, PS4VP-PPIX may be preferential to use to optimize treatment in order to control dosimetry better for patients.

Chapter 5 X-ray activation of Porphyrinated Latex particles by FRET

5.1 Introduction

The FDA approved photosensitizer Protoporphyrin IX (PPIX) stands out amongst a majority of other photosensitizers for applications like photodynamic therapy. Its biocompatibility stems from its development in the mitochondria from the precursor Aminolevulinic acid (ALA). However, exogenous PPIX is still successful in affecting cancer cell treatment. These treatments using PPIX or ALA are typically topical due to PPIX’s capability to absorb red light since red light can penetrate into the dermis of human skin. Interstitial photodynamic therapy also uses red light to treat internal cancer sites by using optical fibers as waveguides. While this continues to take advantage of PPIX’s ability to accumulate more at tumor sites compared to normal cells with using red light to penetrate tissue, interstitial photodynamic therapy is still slightly invasive and it can affect only as deep into tissue as red light can penetrate. An alternate light source that can better penetrate tissue is X-ray. However, free molecular PPIX has low to negligible stopping power for X-ray in comparison to organic scintillators. Therefore a system to enable better PPIX reactions by using X-rays would be beneficial for non-invasive photodynamic therapy. In this paper, we embed the organic scintillator 2, 5-diphenyloxazole (PPO) into the latex particle poly (styrene-co-4-vinylpyridine) (PS4VP), attach PPIX to the scintillator particle’s surface, and employ the Forster Resonance Energy Transfer mechanism to allow energy transfer from PPO to PPIX and bring about visible photoreactions such as fluorescence and singlet oxygen generation.
5.2 Materials and Experimental

Chemicals from Sigma Aldrich included the following: Styrene (Sty) and 4-vinylpyridine (4VP) were used as monomers. Benzoyl peroxide (BP) was used as the crosslinking radical initiator. Sodium dodecyl sulfate (SDS) was used as the anionic surfactant and Protoporphyrin IX (PpIX) was used as the porphyrin photosensitizer. Singlet Oxygen Sensor Green (SOSG) was purchased from Thermo Fisher. The DI water was used after having gone through a double purification system. All solvents and organic chemicals were of reagent grade.

5.2.1 Synthesis of PPO-Encapsulated PS4VP

To embed PPO into PS4VP nanoparticles, a bottom-up heated free radical emulsion polymerization procedure was used. The oil phase consisted of the organic scintillator PPO mixed with the monomers Sty, 4VP, and the initiator benzoyl peroxide. The aqueous phase consisted of a solution of dissolved SDS in doubly purified DI water. The aqueous phase is then stirred and heated to 90° C. After the aqueous phase reaches the desired temperature, the oil phase containing PPO is added dropwise to the heated and stirring aqueous phase. At the heated temperature, the radical initiator auto-ignites and initiates the polymerization of the particles. During the procedure, a change from opaqueness towards yellow transparency in the solution can be observed, indicating the synthesis is ongoing. Under UV exposure, the blue fluorescence from PPO can be seen.
5.2.2 Attachment of PPIX to nanoparticle surface

To complete the final particle, after the solution of nanoparticles has cooled to room temperature, PPIX is dissolved into the solution using a bath sonicator. PPIX is normally extremely hydrophobic, but when adding in to the particle solution, PPIX is seen to sink and slowly dissolve into the solution if left unagitated. If the synthesis was successful, PPIX’s red fluorescence can be seen instead of PPO’s blue fluorescence under UV excitation.

5.2.3 UV-VIS spectroscopy and Fluorescence Spectroscopy and XEOL

A Shimadzu UV-2450 UV-vis spectrophotometer was used to observe the absorption spectra of PPO and PPIX. A Shimadzu RF-5301PC spectrofluorophotometer was used to obtain the excitation and emission spectra of the particles, while a RX-650 Faxitron X-ray cabinet system was used to scan for XEOL. Due to high attenuation of X-ray by water, the particle solutions were measured in plates as opposed to cuvettes.

5.2.3 Size and Zeta Potential Measurement

A ZetaPALS DLS detector was used to measure Dynamic light scattering (DLS) and surface charge of the NPS. The particles were suspended in solution and inserted in the machine, and readings were obtained by the machine’s laser light scattering by the Brownian motion of the nanoparticles.
5.3 Results and Discussion

5.3.1 Synthesis and Size Characterization

The completed PS4VP-PPO-PPIX (3P) particle were found to have a size of 286.2 ± 0.76 nm compared to PS4VP size of 40.1 ± 0.16 nm and PS4VP-PPIX size of 49.7 ± 0.17 nm. This may be due to the inclusion of PPO in the synthesis and affecting the particle as a whole. 3P is considered to have a structure similar to that which is shown in Figure 51. PPO is embedded inside PS4VP during the polymerization of the particle. This particle system uses styrene as the main monomer. Research with scintillators involve synthesized bulk styrene polymers to containing and improving materials for radiation detection, and while PPO is hydrophobic, embedding into particles allows PPO to be used in solution forms.

The 4-vinylpyridine was used because of its ability to be synthesized with styrene and its ability to create bonds with different functional groups, either by quarternization or hydrogen bonding. PPIX properties has also been improved for aqueous solution using PS4VP particles. It was shown that PS4VP can keep PPIX from interacting with itself, which would normally cause aggregation in pure water and diminish its absorption capacity. Using PS4VP, this overall effect allows 3P to be highly suspendable as an aqueous solution.
Figure 51 Schematic for PPO embedded PS4VP bonded with PPIX

During synthesis, particle solutions were observed under UV light. As seen in Figure 52, when keeping proper measurements, effects of the encapsulation of PPO and attachment of PPIX can be seen. Embedded PPO emits a stronger blue fluorescence than simply the synthesized PS4VP particles, and after attaching PPIX, the particles containing PPO seem to give a more intense red emission from PPIX.
5.3.2 Forster Resonance Energy Transfer with XEOL

After synthesis, verification of FRET can begin by first observing the absorption and emission spectra of the donor and the acceptor. One of the conditions for FRET is that the emission spectrum of the donor must overlap with the absorption spectrum of the acceptor. This would be an indication that energy from the excited donor can transfer over to the acceptor, thereby placing the acceptor into an excited state and then allowing and induced fluorescence via energy transfer. Figure 53 shows a FRET diagram of how an excitation of an acceptor can be induced by a donor. This mechanism involves a dipole-dipole coupling mechanism where the energy of the donor transfers to the acceptor. In Figure 54, the emission of PPO peaking at 374 nm overlaps greatly with PPIX's absorption peaking at 412 nm.
Figure 53 Diagram of electron transitions during FRET. If an acceptor’s absorption capabilities are within the emission of a donor, FRET will have a better chance of occurring.

Figure 54 PL spectra showing overlap of PPO emission with PPIX absorption. The shaded region shows adequate overlap of PPO emission with PPIX absorption.
The second condition for FRET to successfully occur is the distance of the donor and acceptor. The reliability of FRET depends on the distance to be very close, since effectiveness is inversely proportional to their distance to the sixth power. With PPO embedded inside the particle's matrix and PPIX attached outside the particle, the distance between the two should be at the angstrom to sub-nano distance. The X-ray excited optical luminescence (XEOL) spectra of PS4VP-PPO and PS4VP-PPO-PPIX, which is shown in Figure 55. The peak at 415 nm of PS4VP-PPO’s spectrum is most likely the emission from PPO. This seems to have red shifted from the 374 nm seen from PPO’s PL spectra when previously dissolved in organic solvent.

In the PS4VP-PPO-PPIX spectrum, four peaks stand out. The peaks at 390 nm and 458 nm seem to be within the region of PPO’s emission. The valley between them with the trough minimum around 417 nm seems to indicate a quenching of PPO emission. The other two emission peaks around 640 nm and 701 nm is the emission from PPIX, with the former emission slightly redshifted from the one seen from its PL spectrum. PPIX emission intensity is found to be 53% compared to PPO’s emission. This evidence is the indication that FRET occurs with this nanoparticle system, where the energy from X-ray that excited PPO goes through a non-radiative transfer to PPIX.
Figure 55 XEOL Spectra of PS4VP-PPO with and without PPIX. The PPO emission peak at 412nm can be seen from PS4VP-PPO particles. For PS4VP-PPO-PPIX, two peaks from PPO’s emission and two peaks from PPIX emission can be seen. The simultaneous detection of the quenching of PPO emission at 412 nm, which resulted in the two peaks at 386 nm and 456 nm and emissions at PPIX signifies energy transfer from the excitation of PPO by X-ray to PPIX.

5.4 Conclusion

The organic scintillator PPO that has been typically used for radiation detector synthesis is used in a nanoparticle system for activation PPIX with X-ray. The synthesis using these hydrophobic molecules and using styrene-based monomers with SDS resulted in particles with increased dispersibility and retained characteristic fluorescence. Spectral data of PPO and PPIX indicated a possibility of FRET to occur, and upon monitoring XEOL, a quenching of PPO and emission of PPIX was observed. The particles’ increase in water solubility and dispersibility with the ability to be activated by X-ray show a possibility in biological applications such as deep tissue PDT. Further study must be done to detect singlet oxygen generation and photodynamic activity in biological systems. Aside from the
porphyrin, this PS4VP-PPO’s particle system shows potential for energy transfer applications. Chemicals with –COOH groups may benefit from the surface provided by this latex particle.

Chapter 6 Conclusion and Future Works

Nanoparticles have been proposed and synthesized to improve PPIX’s properties. Absorption and photoluminescence emission have been retained as seen with PPIX dissolved in organic solvents. The two characteristics were enhanced after PPIX was attached to PS4VP. In vitro studies showed the particles to have good dark toxicity, singlet oxygen generation, and PDT action. Also, the photobleaching process where the reduction of PPIX emission and absorption and the increase of PP emission and absorption were seen in aqueous solution. With overcoming water’s interference, the organic scintillator PPO was added to the synthesis to create an X-ray excitable porphyrin nanoparticle. XEOL data showed FRET occurring with energy being transferred from PPO to PPIX. The enhancement of PPIX properties in water while retaining characteristic properties as seen in typical PPIX studies and the synthesis of the X-ray excitable particle shown the applications of PPIX and the PS4VP particle.

In the future, more singlet oxygen studies will be conducted to compare and explain the efficiencies of the particle system with different electromagnetic radiation. The extent of the bonding capabilities of PS4VP will be further studied to determine if other chemicals can take the advantage of being attached to a nanoparticle’s surface. More in vitro studies will be conducted to determine the efficiency of using UVA and X-ray with PS4VP-PPO-PPIX to kill cancer cells.
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Biographical Information

Brian Bui is a native born Texan of Vietnamese descent. He grew up with the gracious help, endless perseverance, and overflowing love from his mother and father. He received a BS in Biophysics and BA in Mathematics from St. Mary’s University in San Antonio, TX, and he received his Ph. D. in Applied Physics with a focus in luminescent nanoparticles fabrication at the University of Texas at Arlington. He has also received the James L. Horwitz Physics Scholarship. Brian is interested in doing research in nanoparticle synthesis and using nanoparticle systems to improve known applications including biological, chemical, physical, and medical. He has worked on enhancing Protoporphyrin IX for PDT, deep tissue PDT, and experiments in aqueous solutions. He has also published and been involved with literature listed below. In the future, Brian plans to continue doing research while introducing up and coming scientist to interdisciplinary studies and research in the academic setting.

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