Enhancement of Protoporphyrin IX Performance in Aqueous Solutions for Photodynamic Therapy

Homa Homayoni^{b, †}, Ke Jiang^{a, §}, Xiaoju Zou^a, Marius Hossu^a, Leila Hossein Rashidi^{a, b}, Wei Chen^{a}*

^aNano-Biophysics lab, Department of Physics, University of Texas at Arlington, Arlington, Texas 76019, USA

^bJoint Biomedical Engineering Program, University of Texas at Arlington, Arlington, TX, 76019, and University of Texas Southwestern Medical Center, Dallas, Texas, 75235, USA

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ABSTRACT. Molecular modification of protoporphyrin IX (PpIX) was conducted to improve its water solubility and therapeutic performance for photodynamic therapy. The carboxylic acid and the two nitrogen atoms in the core of PpIX molecule were protonated following by conjugation with 3-aminopropyl triethoxysilane (APTES). Then, folic acid (FA) was conjugated to the APTES-coated PpIX (MPpIX) through chemical bonding between FA and protonated PpIX. The results showed that APTES coating can stabilize PpIX and increase its water solubility. Consequently, this leads to the enhancement in luminescence and singlet oxygen production. Upon X-ray irradiation, singlet oxygen can be detected in the MPpIX but not in PpIX. This means that MPpIX can be used for deep cancer treatment as X-ray can penetrate deeply into tissue. Molecular modification also reduces the dark toxicity of PpIX and increases their cell uptake. All these traits indicate that the Molecular modification of PpIX may potentially improve the efficacy of photodynamic therapy for cancer treatment.

Corresponding author:

*E-mail: weichen@uta.edu Phone: (+1) 817-272-1064. Fax: 817-272-3637

Present Addresses

[†]Department of Chemical Engineering, Northeastern University, Boston, Massachusetts 02115, USA

[§]University of Colorado, Colorado Springs, Colorado 80918, USA

INTRODUCTION. Photosensitizer (PS) plays a key role in the efficacy of photodynamic therapy (PDT)¹. Absorbed photons can transform the PS from its ground state (singlet state) into the excited state (triplet state). The excited triplet is followed by two different kinds of reactions. In the type I reaction, the direct reaction of the excited triplet with available molecules or substrate (cell membrane) transfers an electron to form radicals. In the type II reaction, energy transfers from the triplet state of PS to oxygen that can produce singlet oxygen. Both reactions occur simultaneously and the products are toxic to cancer cells²⁻⁷.

The efficacy of PS, light, and tissue oxygen determine the PDT efficacy to kill cancer cells^{1, 8, 9}. Among all available PS drugs, porphyrin-based PSs have been approved clinically in the United States for PDT¹⁰. PpIX has been recognized as a preferable drug because it shows good accumulation in tumor cells due to the reaction deficiency of ferrochelatase enzyme in tumor cells comparing to normal cells. Ferrochelatase enzyme converts endogenous PpIX into heme¹¹⁻¹⁴. In addition, PpIX can be excreted in less than 24 hrs and surplus PpIX may be excreted into the intestine¹⁵. PpIX may be delivered to cells through the escape from lysosome enzymatic degradation due to their potential of photochemical internalization (PCI)^{16, 17}. Even though Photofrin-based photosensitizers has been approved by FDA for clinic applications, it has some issues for practical applications. One challenging issue is its hydrophobicity and low solubility in aqueous solutions. Due to the low solubility (~1 µg/mL) of PpIX in water, its accumulation in skin is high and this may cause a prolonged light sensitivity lasting for up to weeks after PDT treatment. The aggregation of hydrophobic PpIX in aqueous media subsequently undermines efficiency of singlet oxygen production which reduces the treatment efficacy^{9,}

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The administration of large dosage of PS is a current solution for the aggregation and low solubility of PpIX in a physiological medium to achieve satisfactory phototherapeutic windows and clinically relevant cellular levels^{15, 18, 19, 21}. This method, however, has not been appreciated as an efficient resolution as it may increase some drawbacks and side effects related to PpIX aggregation and toxicity such as nausea, vomiting, hypotension combined with marked vasodilatation, and the hypersensitivity of both the skin and the eyes to light. Regarding the PpIX fluorescence that is dose-dependent, however, this does not mean that the increased concentration of PpIX would increase the luminescence intensity or have a better light-induced cell death²²⁻²⁵. Taking all these into account, it is essential to improve PpIX solubility and stability in aqueous solutions.

The goal of this study is to modify PpIX in order to increase its water solubility and stability so to improve its therapeutic effect in cancer treatment. The Molecular modification may increase the biological interactions such as cell uptake, and

consequently, may improve the performance of PpIX on cancer treatment²⁶. The modification of hydroxyl-bearing molecules using organo-silane agent (aminopropyl) triethoxysilane (APTES) may enhance their cellular adhesion property^{19, 26-28}. For that, PpIX is modified with APTES. PpIX is covalently bonded to the amine-terminated APTES surface by a nucleophilic acyl substitution reaction. Molecular modification of PpIX was done through the chemical amide bond between protonated and activated COOH groups of PpIX dichloride and NH₂ groups of APTES which resulted in coating PpIX with a layer of APTES or modified PpIX (MPpIX)^{19, 29}. The modification is expected to improve the solubility and stability of PpIX in the aqueous solutions. Also, the modification is expected to prevent PpIX aggregation, prolong the blood circulation, improve singlet oxygen production, cell uptake as well as the therapeutic effects on cancer treatment.

EXPERIMENTAL SECTION. All organic chemicals and solvents were of reagent grade. Dimethylformamide $\geq 99.9\%$, protoporphyrin IX $\geq 95\%$, (3-aminopropyl)triethoxysilane 99% (APTES), Oxalyl Chloride, N-Hydroxysuccinimide (NHS) and ethyl(dimethylaminopropyl) carbodiimide (EDC), trypsin, fetal bovine serum (FBS; 10X) and Penicillin/Streptomycin, paraformaldehyde (PFA) as a fixing reagent, and Folic acid all were purchased from Sigma-Aldrich. MTT Assay reagent, PBS, and DMSO as a MTT detergent reagent were purchased from Trevigen Inc. DMSO as a solvent, PC3 cells (number CRL-1435), and mounting medium with DAPI were purchased from VWR International, ATCC, and Vector Company respectively.

Preparation of PpIX dichloride. Preparation of activated PpIX shown in Scheme 1 was done by adding 1 mL oxalyl chloride (OC) to 10 mg of PpIX (1.65×10^{-5} mol). The solution was simultaneously stirred and refluxed under nitrogen atmosphere at 55 °C overnight.

Preparation of modified PpIX (MPpIX). As illustrated in Scheme 1, APTES-capped PpIX was obtained by adding 2 mL APTES to the purple powder of PpIX dichloride by simultaneously stirring and refluxing under nitrogen atmosphere overnight. 1 ml Dimethylformamide (DMF) was added as a catalyst. Under a dry nitrogen atmosphere dimethyl-8,13-divinyl-3,7,12,17-tetramethyl-21H,23H-porphine-2,18-dipropyl-midepropyltriethoxysilane was produced as a APTES-capped PpIX which is defined as modified PpIX (MPpIX).

Folic acid conjugated MPpIX (FA-MPpIX). To prepare hydrophilic and tumor-targeted PpIX, FA was conjugated to MPpIX as shown in Scheme 2. In order to prepare FA-conjugated MPpIX, first, 23.39 mg FA (0.053 mmol) was dissolved in 3 mL Dimethyl sulfoxide (DMSO), then 27.47 mg 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 20.37 mg N-Hydroxysuccinimide (NHS) (0.177 mmol of EDC and NHS) were added, respectively. MPpIX (10 mg,

0.0177 mmol) was added into DMSO solution, and the solution was stirred for 3 days. To get rid of all unreacted chemicals, final solution was dialyzed against DMSO/water: 1/10 solution for 3 days (Fisher brand Regenerated Cellulose Dialysis Tubing, MWCO 12000-14000) followed by three times washing in water by centrifuge (Labnet Hermle Z323 Centrifuge device rpm=13500, 25min)^{25, 30-32}.

Characterization. Raman spectra (Horiba Jobin Yvon LabRAM Aramis Raman Spectrometer) were measured on FA, PpIX and FA-MPpIX to confirm the successful formation of FA-MPpIX.

Luminescent properties. The luminescence spectra of PpIX and MPpIX suspended in water at different concentrations starting at 50 µg/mL were recorded by a Shimadzu spectrofluorophotometer (Shimadzu RF-5301PC).

Solubility and stability. Solubility and stability improvement of MPpIX compared to the uncapped sample were observed by dispersing the same concentration of drug for both free PpIX and MPpIX in water for 2 hours; also the same amount of PpIX was dissolved in DMF as a control.

Singlet oxygen generation. As discussed previously, singlet oxygen generation is a key role of PS drugs to kill or damage cancer cells³³, and from this point of view it was important to prove that the proposed MPpIX has been able to improve singlet oxygen generation. A p-nitrosodimethylaniline (RNO)-imidazole (ID) method was used to detect singlet oxygen. Briefly, particles was added into 3 mL of RNO (50 µM) and ID (8 mM) aqueous solution. The intensity of RNO absorption was monitored with various X-ray doses (0, 1, 2, 3, 4, 5, 6, 7, and 8 Gy) using a UV-Vis. Meanwhile, as a reference, the same measurement was done in the absence of drugs to reveal the X-ray irradiation effect on RNO absorption³⁴.

Cell viability of PC3 prostate cells exposed to PpIX, MPpIX, and FA-MPpIX. Cell viability was performed for PC3 cells exposed to different concentrations of PpIX, MPpIX, and FA-MPpIX in order to investigate the effect of APTES coating and FA conjugation on the survival of healthy cells. Cell viability was tested by 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide assay (MTT assay). PC3 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 100 units/mL penicillin at 37 °C and 5% CO₂. The cells were plated into 96-well-plate inserts at 3000 cells per well and allowed to grow for 1 day. The cells were washed with PBS following by replacement of the medium, and then were exposed to different concentrations of PpIX, MPpIX and FA-MPpIX equal to 2.5, 5 and 10 µg/mL of free PpIX for 1 day prior to MTT assay.

Cell uptake and imaging enhancement by molecular modification. In vitro imaging was performed to observe the effects of molecular modification on cell uptake and imaging. If the molecular modification can improve water solubility and stability, it should be able to be excited by light to emit bright red emission for a better imaging. Enhanced red emission will transfer more energy to generate more singlet oxygen^{9, 19, 35}. To observe the drug uptake by cells, PC3 prostate cancer cells were grown on a microscope cover slide and incubated overnight with PpIX, MPpIX, and FA-MPpIX equal to 5 µg/mL free drugs in F-12K medium. The cells were then fixed with formaldehyde (5% in PBS), transferred to a microscope slide for florescent microscopic studies. Images were captured with a florescence microscope (Olympus 1X71 inverted fluorescence microscope) with excitation at 350 nm and emission at 450 or 670 nm.

RESULTS and DISCUSSION

Raman Characterization. Raman spectra were measured on PpIX, Modified PpIX (MPpIX), and FA conjugated to MPpIX (FA-MPpIX) to observe the possible changes before and after molecular modification. As shown in Figure 1, the formation of the siloxane and amide bonding is evidenced in the Raman spectra of FA-MPpIX as compared to that of PpIX. The Raman peak at 600 cm⁻¹ is an indication of the bond formation of Siloxane bridge between PpIX and APTES. The peak at 1200 cm⁻¹, labelled with the black arrow 1, is an indication of both C-N bonding of APTES and (Cb-Cα) stretching of PpIX³⁶⁻³⁹. The peak at 1500 and 3300 cm⁻¹ (labeled with black arrows 2 and 3, respectively) are from the carbonyl and the N-H bonds, respectively. These can attribute to the amide bond formation between the amine (NH₂) groups in APTES or FA and the COOH groups in PpIX. The peak at 1459 cm⁻¹ is a characteristic signal for folic acid corresponding to the phenyl ring. The occurrence of this peak in the Raman spectrum of FA-MPpIX is an evidence for the successful conjugation of FA to MPpIX⁴⁰⁻⁴².

Luminescent properties of MPpIX. The photoluminescence spectra of PpIX and MPpIX are shown in Figure 2, and Figure 3. Both PpIX and MPpIX have two main emission peaks around 620 and 670 nm. Fluorescence of PpIX is typically quantified by the emission around 620 nm while band around 670 nm is considered as a characteristic of the hydroxylaldehyde chlorin photoproduct⁴³. As seen by Figure 2 and Figure 3, the luminescence intensity of both PpIX and MPpIX in water are concentration dependent. For MPpIX, the optimal concentration for the strongest luminescence is at 12.5 µg/mL while for the PpIX the optimal concentration for the highest luminescence is at 25 µg/mL. At the same concentration, the luminescence from MPpIX is much stronger than that of PpIX as shown in Figure 3. The luminescence enhancement in MPpIX is attributed to the improvement of their water solubility and stability. PpIX has a

large hydrophobic core and two hydrophilic side chains. These two groups have a high tendency to form chemical bonds at intramolecular-level between NH group of core and COOH group of side chains. This is why PpIX is easy for aggregation in aqueous solutions. Molecule modification here blocks these hydrophilic side chains through coating PpIX with APTES and reduces the tendency of core and side groups to form chemical bonds. As a result, less aggregates will be formed and energy can be transfer to the single molecule of PpIX to emit more intense luminescence. It is also noted that PpIX spectra has a bump at 686 nm while this tail is not seen in the MPpIX. This bump is likely from some defects or surface states in PpIX, after molecular modification, these defects or surface states are passivated, so this bump at 685 nm disappears. The reduction of defects or surface states by molecular modification is one of the reasons for the luminescence enhancement because the quenching through energy transfer to these defects or surface states is mitigated. The enhancement in the luminescence provides the opportunity for using the photosensitizers not only for therapy but also for imaging.

Solubility and stability of MPpIX. Figure 4 illustrates a good and stable solution of highly concentrated MPpIX (40 µg/mL) in water. In order to compare the stability, PpIX was dissolved in its solvent, DMF, at the same concentration as a control group. The MPpIX water dispersion improvement is clearly observed in Figure 4. In a study by Scolaro et al.⁴⁴ it was demonstrated that the hydrogen bonding involving the carboxylic acid side chains plays a crucial role in PpIX aggregation because of inter and/or intra molecular bonding. They also mentioned that non-aggregated species were caused by protonation of both end groups and the two nitrogen atoms of the core. Thus, it is believed that the substitution of either one of the carboxylic groups through protonation of the PpIX by oxalyl chloride or the incorporation of APTES coating followed by the FA conjugation prevent the aggregation of MPpIX and improve their solubility and stability.

Singlet oxygen generation. The enhancement of luminescence and the water solubility may result in an improvement in the singlet oxygen generation. We are particularly interested in singlet oxygen production induced by X-ray because X-ray activated PDT can be used for deep cancer treatment⁴⁵. Singlet oxygen generation in photosensitizers by X-ray has been investigated but the results reported in literature were controversial^{46, 47}. Some observed high enhancement⁴⁸ while some found that the efficiency of photosensitizers for radiation enhancement is very low⁴⁹. In some cases, photosensitizers even decrease the efficiency of radiation treatment⁵⁰. One possible reason is that the photosensitizers such as porphyrin have very low stopping power and cannot effectively stop or absorb high energy beams like X-ray or Gamma-ray for singlet oxygen production. Once this problem is solved, photosensitizers can be used for deep cancer

treatment. The results shown in the Figure 5 indicate that, upon X-ray irradiation, singlet oxygen is detected in the MPpIX but not in the original PpIX. This means that the modified PpIX can be used for deep cancer treatment. In addition, it can be combined with radiotherapy for cancer treatment, and this combination may enhance the efficacy and reduce the radiation side-effects.

It also should be pointed out that, in general, a photosensitizer would be less effective for singlet oxygen production if it has a strong luminescence. However, for PPIX, the poor water solubility and aggregation in water quench both its luminescence and singlet oxygen production, the increase of its water solubility therefore enhances both its luminescence and singlet oxygen efficiencies.

Observations on dark toxicity. Dark toxicity is an important issue for photosensitizers in practical applications. The reduction of dark toxicity may enhance their biocompatibility. As shown in Figure 6, PpIX is toxic at all examined concentrations with the highest cell survival around 40%. After APTES coating, cell viability of FA-MPpIX is improved up to 70% for the concentration equal to 5 $\mu\text{g/mL}$ of free PpIX. Although with the survival of 70%, it is hard to claim that FA-MPpIX is completely biocompatible, but it should be mentioned that the cell viability has improved almost 30% as compared to free PPIX. Declining aggregation trends of MPpIX because of protonation of both end groups and the two nitrogen atoms of the core of PpIX as well as APTES coating effect can be considered as a major reason of cell viability improvement or the decrease of their dark toxicity.

Cell Uptake Studies: It is expected that molecular modification increases the solubility and luminescence of PpIX, so it would enhance its cell uptake and imaging qualities. Figure 7 displays the cell images when PpIX (left) and MPpIX (right) were incubated with PC3 cells under the same conditions. As shown clearly, the cell uptake is higher for MPpIX. For PpIX, a lot of aggregates were observed, the poor water solubility and the related aggregation are the possible reasons for the poor cell uptake. As displayed in Figure 8, for PpIX uptake by cells no red emission was observed under excitation at 405 nm. However, the FA-MPpIX shows intense red emission under the same excitation as well as higher cell uptakes. These improvements could be due to the modification of PpIX with APTES and FA coating that enhance their water solubility and stability. Meanwhile, over expression of FA receptors on the surface of prostate cancer cells (PC3) have increased the uptake rate. The results of cell imaging were in agreement with previous mentioned results that confirm both the enhancement of photoluminescence and the water dispersion.

CONCLUSION

The poor water solubility, weak luminescence and aggregation of PpIX are the challenging issues for photodynamic therapy on cancer. In this study, we report the improvement of PpIX water solubility and stability by molecular modification. The coating of APTES on PpIX largely increases its water dispersion, stability and luminescence efficiency, as a result, the singlet oxygen production. Our results show that the substitution of either one of the carboxylic groups through protonation of the PpIX using oxalyl chloride and then incorporation of APTES coating and FA conjugation could prevent the aggregation of PpIX. Consequently, the luminescence, water solubility, singlet oxygen production and cell uptake are improved. These results open new door to improve photosensitizers for photodynamic therapy on treating cancers.

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Note:

The authors declare no competing financial interest.

ABBREVIATIONS. APTES, 3-aminopropyl triethoxysilane; NH₂, amine; CE, conjugation efficiency; COOH, carboxyl groups; DMF, dimethylformamide; DMSO, Dimethyl sulfoxide; FA, folic acid; I.V., intravenous injection; MPpIX, Modified PpIX; OC, Oxalyl Chloride; PDT, Photodynamic therapy; PS, Photosensitizer; PpIX, Protoporphyrin IX; PCI, photochemical internalization; UV-Vis, Ultraviolet–visible spectroscopy.

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Captions

Scheme 1. MPpIX fabricated by a two-step chemical process (OC: Oxalyl Chloride)

Scheme 2. Conjugation of folic acid (FA) to the surface of MPpIX

Figure 1. Raman Spectroscopy of PpIX, FA, and FA-MPpIX

Figure 2. Photoluminescence spectra of MPpIX (A) and PpIX (B) excited at 400 nm

Figure 3. The effect of concentration on red emission intensity at different wavelengths: 622-626 nm (A) and 658-686 nm (B) of PpIX and MPpIX excited at 400 nm

Figure 4. Water dispersion improvement of MPpIX in water as compared to PpIX in Water and DMF

Figure 5. Singlet oxygen generation of PpIX and MPpIX (equal to 3.5 $\mu\text{g/mL}$ of free PpIX)

Figure 6. Cell viability of PC3 exposed to PpIX, MPpIX, and FA-MPpIX for 24 hrs tested by MTT

Figure 7. Cell uptake images for PpIX (left) and MPpIX (right) incubation with PC3 cells under the same conditions.

Figure 8. Cell images of PC3 exposed to PpIX (A), and FA-MPpIX (B) recorded on Olympus 1X71 microscope with excitation at 350 nm and emission at 450 or 670 nm. Nuclei was stained with DAPI.

Table of Contents Graphic

Scheme 1

Figure 1

(A)

(B)

Figure 2

(A)

(B)

Figure 3

PpIX
water

MPpIX
water

PpIX
DMF

Figure 4

Figure 5

Figure 7

Figure 8**Merged****Stained Nucleic****Bright Field** **(A)****(B)**