The Development of a Novel Series of Cationic Porphyrins as Disinfectants for Use in Public Health

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Abstract

Aaron Jamal Young: The Development of a Novel Series of Cationic Porphyrins as Disinfectants for Use in Public Health.
(Under the direction of Dr. Louise M. Ball)

In the United States, alone, an estimated 4 million to 33 million cases of gastrointestinal illness resulting from contaminated water supplies occur annually. There is a need for the exploration of new types of disinfectants for water treatment with different mechanisms of action that can be used along with or in place of currently used disinfectants to further improve modern drinking water treatment.

Photodynamic inactivation (PDI) of pathogens is a unique approach to water treatment. In general, PDI consists of a chromophore that absorbs energy from light, and ultimately uses that energy to inactivate pathogens via singlet oxygen. Cationic porphyrins are one group of chromophores that have proven to be effective in the inactivation of viral, bacterial, fungal and parasitic pathogens. It is believed that the positive charge on cationic photosensitizers (PS) help them to better associate with the predominantly negatively charged surfaces on pathogens most resistant to chemical disinfection.

The cationic porphyrins used in previous tests have carried fixed positive charges on the periphery of the tetrapyrrole macrocycle. Porphyrins that carry positive charges connected farther from the ring through an aliphatic carbon
chain have been synthesized and characterized using proton nuclear magnetic resonance ($^1$H NMR), mass spectrometry (MS), and UV-Visible spectrometry (UV/Vis). The hypothesis was that this would allow for the positive charge to move more freely and possibly better adapt to the shape and negative charge distribution on the surface of target microorganisms. Using literature-based isolation techniques and the double layer enumeration method, the new cation location was observed to increase the porphyrins’ binding to, and subsequent inactivation of *E. coli* and *Salmonella*. Viral binding was not as well correlated to inactivation as that of bacteria. This novel group of porphyrins is also marked by a decrease in the compound’s stability as well as its toxicity in the absence of light. This method of increasing cation flexibility, in theory, could be used to increase the efficiency of PDI of bacteria for other synthetic chromophores.
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Table of Contents

List of Tables...........................................................................................................viii

List of Figures..........................................................................................................x

List of Abbreviations.............................................................................................xii

Chapter

I. Introduction............................................................................................................1

   Review of Literature............................................................................................1

   Specific Aims.......................................................................................................11

   References.........................................................................................................14

II. Porphyrin Synthesis and Determination of Optimal Chain Length for Inactivation.................................................................19

   Background.......................................................................................................19

   Materials..........................................................................................................21

   Methods...........................................................................................................22

   Results.............................................................................................................28

   Discussion.........................................................................................................37

   References.......................................................................................................43

III. Porphyrin Photodegradation and Toxicity .......................................................45

   Background.....................................................................................................45
Appendix 2. Additional $^1$H NMR and MS spectra of porphyrins before and after exposure to light ........................................ 103

Appendix 3. Sample calculations for loss of water during the stability experiment in Chapter 3 ........................................ 109

Appendix 4. Actual values from porphyrin binding in Chapter 4 ......... 111

Appendix 5. A sample HPLC chromatogram from measurements of singlet oxygen production in Chapter 4 ................. 114

Appendix 6. Evidence of linear inactivation .................................... 115

References ....................................................................................... 116
List of Tables

Table 2.1 The percent yield for each reaction in the porphyrin synthesis.................29
Table 2.2 Porphyrin inactivation of undiluted \textit{E. coli}........................................34
Table 2.3 1\mu M porphyrin reduction of diluted \textit{E. coli} .................................36
Table 2.4 MS2 reductions with and without light...............................................36
Table 2.5 MS2 reductions using the commercially available TMPyP .................37
Table 2.6 Comparisons of C3PyP and free chlorine inactivation of \textit{E. coli}......40
Table 2.7 Comparisons of C3PyP and free chlorine inactivation of MS2.........40
Table 3.1 The MS peaks of the four porphyrins and their photoproducts ............54
Table 3.2 The LC50s for each of the porphyrins and their photoproducts ............56
Table 4.1 Inactivation of \textit{E. coli} by fixed and flexible cationic porphyrins........69
Table 4.2 Inactivation of \textit{Salmonella} by fixed and flexible cationic porphyrins...69
Table 4.3 Porphyrins' binding to \textit{E. coli}.......................................................70
Table 4.4 Porphyrins binding to \textit{Salmonella} ...............................................70
Table 4.5 MS2 Inactivation by fixed and flexible cationic porphyrins ...............73
Table 4.6 PRD-1 Inactivation by fixed and flexible cationic porphyrins .............73
Table 5.1 MS2 inactivation by porphyrins both in filtered light and in dark........83
Table 5.2 Time needed to reach 3-log MS2 reductions with and without light filter..........................................................84
Table 5.3 C4PyP inactivation of MS2 at 645 nm..............................................85
Table 5.4 Comparison of our study to previous literature on chlorine disinfection of MS2.........................................................89
Table A1.1 A list of previously documented PDI of frank and model pathogens..........................................................99
Table A3.1. The data collected in order to account for water evaporation during the formation of porphyrin products……………………………………………….110

Table A4.1 The paired t-test values for each porphyrin……………………………………112

Table A4.2 The binding to MS2 and PRD-1 measured as percent fluorescence when compared to controls……………………………………………………113
List of Figures

Figure 1.1 A general diagram of the photodynamic process..........................5

Figure 1.2 An example of a fixed (right) and flexible (left) cationic \textit{meso} substituted porphyrins .................................................................10

Figure 2.1 The structure of a generic \textit{meso}-substituted porphyrin ..............20

Figure 2.2 General scheme for porphyrin synthesis, applied to C3PyP ........22

Figure 2.3 $^1$H NMR of ethyl-4-bromo-butyrate from the spectral database ......30

Figure 2.4 $^1$H NMR of 4-bromo-1-butanol after the LiAlH$_4$ reduction (CDCl$_3$) ...30

Figure 2.5 $^1$H NMR of 4-bromo-1-butanal after the PCC oxidation (CDCl$_3$)....31

Figure 2.6 $^1$H NMR after the condensation step (CDCl$_3$). ........................32

Figure 2.7 $^1$H NMR of the final product C3PyP (CD$_3$OD).............................33

Figure 2.8 The name and structure of porphyrins used in inactivation experiments with \textit{E. coli} and MS2.................................................................35

Figure 3.1 The four porphyrins used in this study and their abbreviations.......47

Figure 3.2 The degradation of C4PyP over time............................................50

Figure 3.3 The absorbance of the porphyrin Soret over time........................51

Figure 3.4 The $^1$H NMR of C3PYP before and after 24 hrs irradiation...........52

Figure 3.5 The $^1$H NMR of C4PYP before and after 24 hrs irradiation..........53

Figure 4.1 The four porphyrins used in this study and their abbreviations.......64

Figure 4.2 The porphyrin binding to bacteriophages MS2 and PRD-1 measured via analysis of the supernatant after ultracentrifugation ...............72

Figure 4.3 The porphyrin binding to bacteriophages measured via the fluorescence of the resuspended pellet after ultracentrifugation ...............72

Figure 4.4 Measurement of singlet oxygen- furfuryl alcohol (FFA) product....74

Figure 4.5 Measurement of furfuryl alcohol residual....................................75
Figure 5.1 C4PyP absorbance compared to filter light absorbance .......... 82
Figure 5.2 Control chromatogram of TProPyP (10µM), FFA (100µM) and IS (100µM) without light ................................................................. 85
Figure 5.3 Chromatogram of TProPyP (10µM), FFA (100µM) and IS (100µM) with 5 minutes exposure to filtered light .................................... 86
Figure 5.4 Chromatogram of overlay of figures 5.2 and 5.3 ................. 86
Figure 5.5 Control chromatogram of FFA and IS with 5 minutes exposure to projector light ................................................................. 87
Figure 5.6 Control chromatogram of FFA and IS without light ........... 87
Figure 5.7 Control chromatogram of FFA and IS with 5 minutes exposure to projector light ................................................................. 87
Figure 5.8 Chromatogram of overlay of graphs 5.5, 5.6, and 5.7 ............ 88
Figure A2.1 ¹H NMR of TProPyP before(a) and after (b) 24 hours of exposure to light ................................................................. 104
Figure A2.2 ¹H NMR of TBuPyP before (a) and after (b) 24 hours of exposure to light ................................................................. 105
Figure A2.3 The mass spectra of C3PyP (a) and its photoproduct (b) .... 106
Figure A2.4 The mass spectra of C4PyP (a) and its photoproduct (b) .... 107
Figure A2.5 The mass spectra of TProPyP before (a) and after (b) 24 hours of exposure to light ................................................................. 108
Figure A2.6 The mass spectra of TBuPyP before (a) and after (b) 24 hours of exposure to light ................................................................. 109
Figure A4.1 The increase in the absorbance of the Soret of TMPyP over time ................................................................. 114
Figure A4.2 The absorbance of the Soret of TProPyP and TBuPyP over time ................................................................. 114
Figure A5.1 A sample chromatogram of the porphyrin, photoproduct, furfuryl alcohol (FFA), and the internal standard ......................... 115
Figure A6.1 Graphs showing linear inactivation of E. coli and Salmonella by C3PyP and C4PyP ................................................................. 116
List of Abbreviations

C3PyP meso- tetrakis (3-[N-pyridiniumyl] propyl) porphyrin tetrabromide
C4PyP meso- tetrakis (4-[N-pyridiniumyl] butyl) porphyrin tetrabromide
C5PyP meso- tetrakis (5-[N- pyridiniumyl] pentyl) porphyrin tetrabromide
C7PyP meso- tetrakis (7-[N- pyridiniumyl] heptyl) porphyrin tetrabromide
C11PyP meso- tetrakis (11-[N- pyridiniumyl] undecyl) porphyrin tetrabromide
CDC Centers for Disease Control and Prevention
CCl4 Carbon tetrachloride
CFU Colony forming unit
CHO Chinese Hamster Ovary
DIW Deionized water
EC3000 E. coli strain C3000
FAmp Ampicillin resistant E coli strain
FFA Furfuryl Alcohol
Hp Hematoporphyrin
HpD Hematoporphyrin derivative
HPLC High performance liquid chromatography
1H NMR Proton nuclear magnetic resonance
MDL Method detection limit
MS Mass spectrum
NTU Nephelometric turbidity units
PBS Phosphate buffered saline
PCA para-chloranil
PCC Pyridinium chlorochromate
PDI Photodynamic Inactivation
PDT Photodynamic therapy
PFU Plaque forming unit
PS(s) Photosensitizer(s)
ROS Reactive oxygen species
SDS Sodium dodecyl sulfate
SN Supernatant
STDEV Standard deviation
TBuPyP Meso-tetrakis (4-[N-butyl] pyridiniumyl) porphyrin tetrabromide
TMPyP tetraakis (1-methy-4-pyridyl) porphyrine tetra- p- tosylate
TProPyP Meso-tetrakis (3-[N-propyl] pyridiniumyl) porphyrin tetrabromide
TSA Tryptic soy agar
TSB Tryptic soy broth
UV/Vis Ultraviolet to visible light spectrum
Chapter 1: Introduction

Review of Literature

After 100 years, disinfection remains a cornerstone of modern water treatment. Although current water treatments, such as chlorine, are relatively effective, there are still some inadequacies. In the United States alone, an estimated 4 million to 33 million cases of gastrointestinal illness resulting from contaminated water supplies occur annually. New challenges, such as biological contamination of water sources, an aging water distribution system, and increasing water reuse will require us to remain vigilant in protecting all aspects of our water supply. Many pathogens, such as various parasites, gram-negative bacteria, and non-enveloped viruses, have proven to be resistant to currently used disinfectants for water treatment. This along with any additional biological contamination from surface runoff, flooding, and/or the increase in water reuse, could lead to both temporary and perpetual inadequacies in drinking water treatment.

Scientific breakthroughs and improvements in water safety regulations are needed to ensure that communities continue to receive safe drinking water. There is a need to explore new types of disinfectants for water treatment with different mechanisms of action that can be used along with or in place of
currently used disinfectants to further improve water quality in developed countries.

This belief is strongly supported by the U.S. Centers for Disease Control and Prevention (CDC)\(^4\). One of the CDC’s major goals is that people be prepared for emerging health threats. Their literature states the following:

> [one CDC goal is to] “Support and strengthen human and technological epidemiologic resources to prevent, investigate, mitigate, and control current, emerging, and new public health threats and to conduct research and development that lead to interventions for such threats.”

So, new methods of disinfection should be developed in order to protect people, not only from current waterborne pathogens, but also to protect us from new emerging health threats due to drinking water.

Photodynamic inactivation (PDI) of pathogens is a unique approach to water treatment. PDI, in general, consists of a chromophore that absorbs energy from light, and ultimately uses that energy to inactivate pathogens via singlet oxygen. One method of PDI, called Photodynamic Therapy (PDT), is an approved treatment for several cancers\(^5^9\). While PDI and PDT both rely on the same photodynamic processes, in the present work, PDI is used to describe the application of those processes to disinfection where PDT refers to the cancer treatment.

The use of photodynamic processes is not a new phenomenon by any means. Much of the recent work using photosensitizers (PSs) has been geared toward developments in PDT, yet many of the findings are still relevant to PDI.
Furthermore, the use of synthetic porphyrins, our PS of choice, was originally developed for PDT of cancers.

The oldest documented use of PSs was over 3000 years ago in India, where psoralens and light were used to treat vitiligo15. In 12th century AD, Egyptians also used psoralens as a treatment for leucoderma8. Modern methods of PDI/PDT began to emerge in the 1900's. In 1903 Herman Von Teppeiner used eosin and sunlight to treat skin tumors in mice. In 1907 he also showed that oxygen was required for the photodynamic killing of tumor cells. Similar observations were made with chlorophylls and erythrocyte hemolysis in 1907 and 19198. By 1911, experimentation on the photosensitization of mice with hematoporphyrin (Hp), a compound naturally found in the body, was well underway6,16. In 1913, Hp was found to cause photosensitization in man when Friedrich Meyer–Bertz injected himself with 200 mg of Hp and observed no adverse effects until he was exposed to light6,8,16.

In 1924, it was found that natural porphyrins in tumors could give off fluorescence6,8,16, while in 1942, Hp was found to accumulate in tumors. This ability of the natural porphyrins to accumulate in tumors and give off fluorescence was first developed to detect and quantify tumor growth. In 1960-61, Hp derivatives (HpD) were synthesized and used for tumor detection17. HpD would be used for tumor detection throughout the 1960’s.

Throughout the 1970’s, Hp and HpDs were developed for PDT. Tumors were first successfully treated in rats18,19, which led to the first clinical trials of HpD by the end of the decade20-22.
To date photosensitizers activated by light are being used as a treatment to stop uncontrolled cell growth. PDT is an accepted treatment for macular degeneration as well as several types of cancer\(^5\)\(^-\)\(^9\). In PDT the photosensitizer is delivered to the target area and activated, often with lasers, to bring about a cytotoxic effect. PSs and light have been shown to kill all classes of pathogens: bacteria, viruses, parasites and fungi\(^23\)\(^-\)\(^26\). Although PDI has been documented in various aqueous media, such as natural waters and blood plasma, its application to drinking water is relatively new.

There are two types of photodynamic processes in PDT/ PDI. In the type I process, the excited PS directly undergoes hydrogen or electron transfer with biological substrates that, when oxidized, impairs some cell function. The type II process involves the excited PS transferring energy to dissolved oxygen to produce reactive oxygen species (ROS), most likely singlet oxygen, which then oxidizes biological substrates. See Figure 1.1.
Figure 1.1 A general diagram of the photodynamic process ⁸.

*Sₙ represents the various energy levels, and T₁ represents the excited PS. This figure was obtained from Pushpan et al. ⁸.

The efficiencies of both PDI and PDT are dependent on the following PS properties ⁹:

I. Lipophilicity and ionization
II. Molar extinction coefficient
III. Quantum yield of triplet state
IV. Redox potential of the excited porphyrin
V. Yield of singlet oxygen

I. Lipophilicity and ionization- The lipophilicity and ionization affect the PS’s association and ultimate incorporation into the target pathogen or cell.

Amphiphilic character can be helpful for PS mobility through cell walls and membranes, as well as through intercellular spaces. The active agent in PDI is
either the excited PS or the reactive oxygen species. Both are very short lived and must be in close proximity to the target to have a biocidal effect.

II. Molar extinction coefficient- The molar extinction coefficient is a measure of the ability of the PS to absorb specific wavelengths of light. The extent of PS activation is proportional to the light energy absorbed by the PS.

III. Quantum yield of triplet state- The Quantum Yield of the triplet state is the amount of excited PS produced relative to the photons absorbed from light. There are two ranges of light that seem most promising in PDI. The visible light range (400-650 nm) is the range of ambient light that can be detected by the human eye and electrically. This range allows for inexpensive light sources in which the PDI could take place in a natural environment supported by sunlight. The second range of long wavelength light (650-900 nm), although much more expensive to produce, can allow for deeper penetration into tissues (PDT) as well as through turbid waters (PDI). In terms of drinking water treatment, the disinfection processes generally take place after coagulation and flocculation, which removes most, if not all, of the turbidity.

IV. Redox potential of the excited porphyrin- The redox potential of the excited PS is valid for the type I process in which the PS directly oxidizes biological substrates of the target pathogen or cells. The redox potential is a measurement of how well the excited PS could oxidize those substrates.
V. Yield of singlet oxygen - The yield of singlet oxygen pertains to the type II reaction, in which the excited PS transfers its energy to molecular oxygen to produce the biocidal singlet oxygen that inactivates pathogens. Singlet oxygen is believed to be the active ROS in PDI.

Other important PS characteristics needed for the practical application of PDI include an economic synthesis, low native state toxicity, and rapid elimination from the body. PSs used in water treatment will undoubtedly have to compete with the most inexpensive methods of disinfection, such as chlorine, which will require a reasonably simple and inexpensive synthesis. If used in drinking water treatment, some amount of the PS will be ingested, which will require the PS to be both nontoxic in the absence of light and rapidly eliminated from the body to reduce the potential adverse health effects due to PDI.

Since the development of Hp and HpDs for cancer treatment, several other PSs, naturally occurring and synthetic, have been selected to try to increase the efficiency of PDT based on the above-mentioned characteristics of a good PS. Each PS still has some drawbacks and there has not been one PS that is accepted as an agent for all PDT. In both PDT and PDI, increasing the PS association with target cells and/or pathogens is key; close association with the target pathogen is important given the short-lived ROS ultimately responsible for inactivation. Singlet oxygen, which is thought to be the biocidal species in most photodynamic processes, has a lifespan of 100-250 ns and an estimated diffusion distance of roughly 50 nm in aqueous media. So, as a result, the
active agent will travel less distance than the diameter of the target malignant
cells in PDT and the bacterial cells in PDI. For effective killing of both tumor cells
and pathogens, the PS’s association with the target is likely equally or more
important than its overall production of singlet oxygen.

Much of the latest developments in PDT have been geared toward the
production of PSs that specifically target malignant cells so as to reduce the
generalized killing of healthy cells. In general, PSs bound to conjugates for
receptors on target cells have been used to increase the efficiency of PDT. This
selectivity both increases the toxicity to malignant cells and reduces toxicity to
healthy cells in PDT. For example, chlorin-bound microspheres have been found
to increase PDT of human bladder carcinoma cells. Also anti-estrogen
conjugated porphyrins have been shown to increase the efficiency of PDT in
MCF-7 breast cancer cells. Even PSs bound to internalizable ligands and
proteins that are recognized by and actively transported into the cell nucleus
have proven to improve PS association with and uptake by the cell. Subtle
changes to the PS can also result in large increases in efficiency. Small
increases in the aliphatic chain length bound to PSs were shown to increase
lipophilicity of the compound and its uptake by cells.

The charge on the PS also plays a role in its interactions with the cell
membrane. Recent studies have shown that cationic PSs, irradiated with light,
are more efficient than anionic PSs in the inactivation of non-enveloped viruses
and gram-negative bacteria, two types of pathogens that are more
resistant to currently used disinfectants than their gram-positive or non-
enveloped counterparts. It is believed that the positive charge on cationic PSs help them to better associate with the predominantly negatively charged surfaces on non-enveloped viruses and gram-negative bacteria, thus bringing the PS in close proximity to the target pathogen. Furthermore, since the above and other parasitic pathogens, such as Cryptosporidium parvum and Giardia lamblia, that are most resistant to current methods of disinfection all have a net negative charge, cationic PSs are often the focus of PDI.

Using the latest developments in PDT as a model, it would seem that PSs could be developed to specifically inactivate pathogens in drinking water and other aqueous media. Small amendments to a PS’s structure and properties could make the PS more efficient for the PDI of aqueous media.

Porphyrins are one group of chromophores proven to be effective in the inactivation of viral, bacterial, and fungal and parasitic pathogens (See Appendix 1). Porphyrins are naturally occurring in the body and are believed to be less toxic than other PSs with chemical structures that are completely xenobiotic. A cardinal characteristic of porphyrins is their ability to accommodate various substituents bound to the macrocycle. Changing the substituents alters the reactivity of the porphyrin, and can allow for advanced disinfection control and specificity.

Initial tests have shown the porphyrins to be active in the presence of light at concentrations that have no biocidal effect in the dark. This makes the porphyrins very appealing as disinfectants because, if ingested, their toxicity is reduced in the absence of light. This disinfectant with a non-toxic residual, in
theory, could be used for treating various aqueous media such as drinking water and blood products\textsuperscript{10-14}.

Previously synthesized cationic porphyrins that have been proven to inactivate non-enveloped viruses and gram-negative bacteria in aqueous media have had fixed positive charges located on the periphery of the tetrapyrrole macrocycle\textsuperscript{37-41}. To date one study has included a porphyrin with a more flexible cation position; while in this study the flexible cation gave the most efficient PDI, the effect of the cation position was not directly observed\textsuperscript{39}. In the present study, the positive charge is connected farther from the tetrapyrrole macrocycle through an aliphatic carbon chain to give the porphyrin added flexibility to associate with the negative regions on the surface of the target pathogens (See figure 1.2).

**Figure 1.2** An example of a fixed (right) and flexible (left) cationic *meso* substituted porphyrins.*

![Diagram of fixed and flexible porphyrins](image)

*The newly synthetisized porphyrins (left) vary in the chain length linking the cationic pyridinium. Porphyrins described in previous literature (right) have a more rigid cation position in which the pyridinium is directly attached to the macrocycle.*
A series of porphyrins were tested against model pathogens \textit{E. coli} C3000 and \textit{F Amp}, \textit{Salmonella LT2}, and bacteriophages MS2 and PRD-1. MS2 and PRD-1 are examples of non-enveloped viruses, which have ssRNA and dsDNA respectively. MS2 has also been previously documented as a model for the hepatitis A virus.\(^{42,44}\) The inactivation of non-infectious strains of \textit{E. coli} is used to offer understanding of the porphyrins’ efficiency against bacteria commonly used as an indicator of fecal contamination\(^{45}\). Salmonella, which is the host for PRD-1, served as a second representative gram-negative bacteria.

In order for this class of porphyrins to become useful in a public health setting, this group of porphyrins must be characterized for use in water treatment. Specific questions must be answered as to better understand the porphyrins’ stability in storage and in use, native state toxicity, and other factors that influence PDI efficiency.

Porphyrin analogs to the above-described porphyrins that have the positive charge connected at the periphery of the porphyrins’ macrocycle were also synthesized in order to observe the effects of the positive charge location on various properties relevant to PDI. The singlet oxygen production and the final localization of porphyrin associated with the target cells were also measured

\textbf{Specific Aims}

This research was intended to develop the use of a novel series of photoactive cationic porphyrins as disinfectants for fluids such as water and blood products. In order to move forward in implementing their possible applications as disinfectants, a greater understanding of these agents’
mechanism of action, potential for toxicity, and stability in use is needed. A series of experiments was conducted to better define the porphyrins’ applicability to the disinfection of water and other aqueous media and answer the following questions.

(1) **What is the optimum chain length for porphyrin inactivation of model pathogens?** The cationic porphyrins used in previous tests have carried fixed positive charges on the periphery of the tetrapyrrole macro cycle\(^{37-41}\). Porphyrins with a more flexible cation linkage have been synthesized to adapt to various negative charge distributions on the surface of pathogens. These porphyrins have cations attached to the porphyrin through an aliphatic carbon chain. The inactivation of model pathogens by a range of meso substituted porphyrins with the cation positioned at various chain lengths was measured.

(2) **What is the stability of the porphyrins under various conditions that simulate likely real-life use?** There is a need to better understand how long the porphyrin maintains its integrity when irradiated as well as in the absence of light. In this study, the photostability of four porphyrins that differ only in the mobility of the cation was examined. The formation of degradation products was observed using \(^1\)H NMR, UV/Vis and mass spectrometry.

(3) **What is the potential toxicity of the porphyrins and their degradation products?** The toxicity of the parent porphyrins and the photoproducts to model mammalian cells was measured in order to understand the potential adverse effects of porphyrin use. The control data from the inactivation experiments also offered insight to the toxicity of these porphyrins in the absence of light.
4) How does the attachment of the cation to the porphyrin periphery affect the binding to and subsequent inactivation of specific bacteria and viruses? The efficiency of PDI of the above-mentioned porphyrins and their fixed cation analogs was observed to better understand the effect of the flexible cation position. The amount of porphyrin bound to the target microbe was also measured and compared to each of the porphyrins’ inactivation of model pathogens.

(5) Will long-wavelength light excite the porphyrins to a level effective in disinfection? Porphyrins have a maximum absorbance at 411-430 nm, but are able to absorb longer wavelengths of light, up to 650 nm, to a lesser degree. If this longer wavelength light is still able to excite the porphyrins above the threshold for a biocidal effect, these porphyrins could be used for a wider variety of settings that require deeper light penetration of media.
References


Chapter 2: Porphyrin Synthesis and Determination of Optimal Chain Length for Inactivation

Background

Photoactive disinfectants are of great interest as alternative measures against microbial contamination of aqueous media. To date, photosensitizers (PSs) and light have been shown to kill all classes of pathogens; bacteria, viruses, parasites and fungi\textsuperscript{1-4}. While Photodynamic inactivation (PDI) has been used in various aqueous media, its application to drinking water is relatively new.

Porphyrins prove to be an interesting PS for a number of reasons. Perhaps the greatest reason that porphyrins demand closer investigation is the variety of substituents that can be placed on the porphine skeleton. The most familiar porphyrin, found in hemoglobin and myoglobin, is porphyrin IX, which has many substituents. Changing the substituents on the porphyrin can increase the physico-chemical interaction with specific molecules; thus, porphyrins can be tuned to promote PDI.

In general, the pathogens of concern in modern drinking water treatment all have surfaces with a net negative charge\textsuperscript{5}. Cationic substituents are believed to increase both the porphyrin association with the negatively charged surfaces and the photolytic activity against the pathogens of concern. Most of the previously synthesized cationic porphyrins that have been proven to inactivate non-enveloped viruses and gram negative bacteria in aqueous media have had fixed positive charges on the periphery of the tetrapyrrole macrocycle\textsuperscript{6-14}. By
connecting the positive charge farther from the tetrapyrrole macrocycle through an aliphatic carbon chain, the porphyrin flexibility to associate with the negative regions on the surface of the target pathogens is increased (See figure 2.1).

**Figure 2.1** The structure of a generic meso-substituted porphyrin.*

![Generic meso-substituted porphyrin](image)

*n=1 meso- tetrakis(1-methy-4-pyridyl) porhryine tetra- p- tosylate (TMPyP)
n=3 meso- tetrakis (3-[N-pyridiniumyl] propyl) porphyrin (C3PyP)
n=4 meso- tetrakis (4-[N-pyridiniumyl] butyl) porphyrin (C4PyP)
n=5 meso- tetrakis (5-[N- pyridiniumyl] pentyl) porphyrin (C5PyP)
n=7 meso- tetrakis (7-[N- pyridiniumyl] heptyl) porphyrin (C7PyP)
n=11 meso- tetrakis (11-[N- pyridiniumyl] undecyl) porphyrin (C11PyP)

*Our synthetic porphyrins vary in the length of the aliphatic bridge which isolates the cationic center from the tetrapyrrolic macrocycle. The porphyrins used in this study (and their abbreviations) based on the figure are as follows. The TMPyP porphyrin (from previous literature) has a tosylate counterion, whereas the others have bromine.

While there has been documentation of the potential of flexible cationic porphyrins, few studies have observed the effect of the length of the aliphatic bridge between the porphyrin macrocycle and the cationic pyridinium. In pursuit of the first specific aim of this research, the above porphyrins were synthesized
and tested against *E. coli* in order to determine an optimal chain length for porphyrin PDI.

**Materials**

*Porphyrin Synthesis*

Solvents methylene chloride, ether, and dimethyl formamide (DMF), and the reagent zinc acetate dihydrate were obtained from Fisher Scientific. Other solvents chloroform, hexane, ethyl acetate, and methanol were obtained from Mallinckrodt Chemicals. Reagents ethyl 4-bromobutyrate, ethyl 6-bromohexanoate, pyrrole (98%), pyridine (99%) HPLC grade, carbon tetrachloride (99%) HPLC grade, lithium aluminum hydride (95%), trifluoroacetic acid (99%), and pyridinium chlorochromate (PCC) (98%) were obtained from Sigma Aldrich. The p-chloranil (PCA) was obtained from Kodak. Montmorillonite clay K10, florsil 100-200 mesh, filter agent celite 521, alumina gel, standard grade, 50 mesh, 58 Å and silica gel, merck grade 9385, 230-400 mesh, 60 Å were also obtained from Sigma Aldrich. All solvents were evaporated using a Büchi Rotovapor R-114. ¹H NMR spectra were recorded on a Varian INOVA 500 spectrometer at 500 MHz and 25°C, using chloroform- *d* as the solvent. Mass spectra were obtained on a Finnigan LCQ DECA quadrupole ion trap mass spectrometer with electrospray source operated in the positive mode. TMPyP was purchased directly from Aldrich Chemical Co., Milwaukee, WI.

*Inactivation Experiments*

USB Agar was obtained from Amersham International, Cleveland, OH. Bacto tryptic soy broth (TSB), glycerine, and tryptic soy agar (TSA) were
obtained from Becton, Dickinson and Co. *E. coli* strain C3000 was obtained from ATCC 15597. A Phillips 40 W Hg fluorescent light (F40T12/DX), positioned 11.25 inches above the wells (measured by ruler), was used to irradiate samples at 0.48 mW/cm² measured by light meter (Mannix DLM2000).

**Methods**

*Porphyrin Synthesis*

All porphyrins except TMPyP were synthesized in our laboratory according to Figure 2.2 which was adapted from literature methods. The product identity and purity was confirmed by ¹H NMR between each step in the synthesis. The synthesis of the tetrakis(N-propyl) derivative, C3PyP, is described in detail below. Additional ¹H NMR and MS spectra can be seen in Appendix 2.

**Figure 2.2** General scheme for porphyrin synthesis, applied to C3PyP*

*All porphyrins were synthesized according to figure 2.2 using the appropriate ester except TMPyP, which is commercially available.*
1) Lithium Aluminum Hydride (LiAlH$_4$) Reduction$^{16}$

In a 500 mL flask, 15 g ethyl 4-bromobutyrate was combined with lithium aluminum hydride in a 1:1 molar ratio. The hydride was added to 100 mL of ether and refluxed at 40°C while the ethyl 4-bromobutyrate was diluted with 60 mL ether and added dropwise. The stirred solution was refluxed for 1 hour. The mixture was then cooled in ice and 10 mL saturated aqueous sodium chloride was added dropwise. Here the solution’s gray color may yield some white precipitate of LiOH. The LiOH was then removed by filtration under vacuum through a sintered glass funnel, and the clear solution was evaporated to yield a colorless alcohol stored at -20°C. The alcohol was then analyzed using $^1$H NMR before going to the second step of the process.

2) PCC Oxidation$^{19}$

The second step of this synthesis was a PCC oxidation using 3-5 g of the above alcohol in a 1:1.25 ratio with the PCC. Here the PCC was combined with 100 mL of freshly distilled methylene chloride and stirred to uniform suspension. The alcohol from step one, dissolved in 60 mL of methylene chloride, was added and stirring was continued for 1 hr at room temperature. Stirring may slow down or stop toward the end as a result of the formation of thick chromium salts. The mixture was diluted with 100 mL of ether, sonicated, and separated on a 1” x 6” silica column eluted with methylene chloride. The green portion of the eluate was collected and further purified on a 1” x 5” silica column eluted with ether. On the second column the colorless aldehyde was collected as the target compound, confirmed using $^1$H NMR, evaporated, and stored at -20°C.
3) Condensation

The third step of this synthesis was a condensation of the above aldehyde and pyrrole in a 1:1 molar ratio\textsuperscript{17,18}. 3 g of the aldehyde and the corresponding portion of dry pyrrole were mixed with 100 mL of carbon tetrachloride (CCl\textsubscript{4}) under argon for 15 min. 1 mL of trifluoroacetic acid was injected into the mixture as a catalyst. The argon was cut off and the mixture was allowed to stir for 24 hrs at room temperature. The CCl\textsubscript{4} was removed by evaporation, and the dry residue was dissolved in 50 mL of methylene chloride and separated on a 1” x 6” alumina column eluted with chloroform. The eluate was then evaporated to 150 mL and refluxed with 300 mg p-chloranil for 40 min. The solution was separated on a 1” x 6” alumina column eluted with chloroform, evaporated to 10 mL, and further purified on a 1” x 7” silica column eluted with 1:1 hexane to chloroform. Finally, the solution containing the porphyrin freebase was evaporated and stored at -20°C.

A second method for condensation was also used in an effort to achieve a better yield\textsuperscript{15,20}. In this method, 10 g of montmorillonite clay was activated at 120°C and below 0.5 Torr for 2 hrs in a 2 L round bottom flask. Next, the system was filled with argon and shielded from light. 950 mL of freshly distilled methylene chloride and 10 mmol of the aldehyde dissolved in 50 mL of methylene chloride were added to the flask. The mixture was stirred to uniform suspension, 10 mmol of pyrrole was introduced dropwise, and the mixture was allowed to stir for 24 hrs. 7.5 mmol of solid p-chloranil was added to the solution, and it was refluxed at 45°C for 1 hr. The solid was filtered through celite
under vacuum and washed with ethyl acetate. The filtrate was reduced and absorbed onto florisil. The absorbate was then purified by column chromatography though two separate 1” x 6” silica columns using methylene chloride. The solution was then evaporated and stored at -20°C. The solid product was analyzed with $^1$H NMR and UV visible spectroscopy before the next step. The presence of the porphyrin can also be determined by the property of the porphyrin to yield a red fluorescence under long wave UV light.

4) Quaternization

The fourth and final step of this synthesis was a quaternization using the porphyrin produced from the above condensation procedure and pyridine. 50 mg of the porphyrin freebase was combined with 12 mL dry pyridine, and refluxed under argon gas for three hours. The solid pyridinium porphyrin was removed and washed with ether. Excess ether was removed and the solid was dried using a rotovapor, oil pump, and lyophilizer. The final product was confirmed via $^1$H NMR.

A total of five different quaternized porphyrins were synthesized via the above methods. These porphyrins have positive charges connected through three, four, five, seven, and eleven carbon chains. The above describes the synthesis of the C3PyP porphyrin with four propyl substituent chains at the meso positions of the ring. To form the analogous porphyrins with the 4, 5, 7, and 11 carbon substituent chains, ethyl 5-bromovalerate, ethyl 6-bromohexanoate, 8-bromo-1-octanol, and 12-bromo-1-dodecanol were used respectively instead of ethyl 4-bromobutyrate as the starting material.
**Inactivation Tests**

All steps of the inactivation tests except for microbe incubation were performed in a biological safety cabinet. All inactivation experiments were done in triplicate. Only the average, not the individual values of the replicates, are shown in the results section. The Thomas Equation was used to calculate the concentration of both the bacteria and viruses.

The above-mentioned porphyrins were used in experiments to measure their efficiency in killing *E. coli C3000* according to the following protocol. The *E. coli* stocks were made by first inoculating into 30 mL sterile TSB made to the manufacturer’s specifications and then incubating at 37˚C for 18 hrs in a forced air incubator with a shaker. 100 µL of the *E. coli* culture was then re-inoculated in 30 mL of sterile tryptic soy broth and incubated for 4 hrs. This four-hour *E. coli* culture was diluted with glycerine in 3:1 ratio, *E. coli* to glycerin, and divided into 1mL aliquots and kept at -80˚ C until further use.

For each experiment the above was repeated using the frozen stocks for inoculation. The four-hour culture of *E. coli* was diluted with sterile deionized water (DIW) in a 2:1 ratio of water to *E. coli* suspension to make the bacterial test mixture. 1 mL of the *E. coli* mixture was added to a sterile well culture plate along with 10 µL of a 0.1 mM porphyrin solution in sterile DIW. This yielded a final porphyrin concentration of 1 µM. Controls were made with the addition of 10 µL of DIW to 1 mL of the *E. coli* suspension instead of the porphyrin. The entire plate was then placed under fluorescent light for as long as 30 min. Additional
controls made with plates identical to that above, were shielded from all light for 30 min.

Duplicate serial dilutions were made from each well, experimental and control, at 10, 20, and 30-minute intervals. Once the final concentrations were achieved, 100 µL of the each dilution from each well was added to 2.5 mL of sterile molten top agar (0.7% agar) kept at 45°C. The samples were then vortexed, poured onto a Petri dish containing 15 mL of TSA, made to the manufacturer's specifications, and allowed to cool and solidify. The plates were then incubated for 20 hrs in a shelf incubator set at 37°C. After this incubation, the *E. coli* colonies on each of the plates were counted. In most cases there were three consecutive countable 10-fold dilutions. All countable dilutions were used to obtain a value of colony forming units (CFU)/mL.

The porphyrins were also tested against the coliphage MS2. The MS2 stock was made by adding 10 µL of concentrated MS2 (~7 x 10^{11}/mL) and 300 µL of the undiluted four-hour *E. coli* culture to 5 mL top agar made of TSB with 0.3% agar. The top agar was gently mixed and poured onto a large Petri plate containing 25 mL of TSA. The plates were then incubated for 20 hours at 37°C. 5mL of phosphate buffered saline (PBS; pH 7) was then added to each plate, and the loose top layer was scraped off and centrifuged at 4500 rpm for 20 minutes at 4°C. The supernatant was then passed through a 0.45 µm filter, and 1 mL aliquots were made and stored at -80°C until further use.

For MS2 inactivation tests, 10 µL of a 0.1 mM porphyrin solution in sterile DIW was added to 1 mL of the MS2 suspension (~1-4 x10^9 PFU/mL). After one
minute of light irradiation the test mixture was serially diluted. 100 µL of the diluted test mixture was added to 200 µL of the *E. coli* culture, described above, in 2.5 mL of top agar made of TSB with 0.7% agar kept in a water bath kept at 45˚C. This mixture was gently swirled and poured onto a Petri dish containing 15 mL of tryptic soy agar (TSA) made to the manufacturer's specifications and allowed to cool and solidify. These plates were then inverted and incubated for 20 hours at 37˚C. All countable plates were used to obtain a value of plaque forming units (PFU) per mL.

**Results**

*Synthesis*

The results described below are for the synthesis of the C3PyP porphyrin. All of the porphyrins have similar $^1$H NMR spectra, however with each increasingly long substituent chain, an additional peak will occur in or to the right of the region of the C2 substituent carbon described below. In general, the porphyrins become increasingly difficult to purify with the increase in carbon chain length. The $^1$H NMR (for the synthesis of C3PyP) of the starting compound, each intermediate compound and the final product can be seen in Figures 2.3-2.7. $^1$H NMR and mass spectra of the other porphyrins can be seen in Appendix 2. The percent yield for each step can be seen in Table 2.1.
Table 2.1 The percent yield for each reaction in the porphyrin synthesis.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Starting Material</th>
<th>Product</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>LiAlH$_4$ Reduction</td>
<td>10.5g</td>
<td>7.4g</td>
<td>70.5%</td>
</tr>
<tr>
<td>PCC Oxidation</td>
<td>5.4g</td>
<td>3.1g</td>
<td>62.2%</td>
</tr>
<tr>
<td>Condensation (trifluoroacetic acid)</td>
<td>3.7g</td>
<td>22mg</td>
<td>0.6%</td>
</tr>
<tr>
<td>Condensation (clay)</td>
<td>2.85g</td>
<td>620mg</td>
<td>12%</td>
</tr>
<tr>
<td>Quaternization (pyridine only)</td>
<td>10mg</td>
<td>10mg</td>
<td>100%</td>
</tr>
</tbody>
</table>

**LiAlH$_4$ Reduction**

The carbonyl group of the ethyl-4-bromobutyrate was reduced with LiAlH$_4$ to give 4-bromobutanol. This process is done with 60-70% yield. This reduction was performed using different concentrations of LiAlH$_4$ and ethyl 4-bromobutyrate and the more dilute solutions tended to give a better yield. $^1$H NMR spectra were used to confirm the new product and its purity. The most noticeable peaks in the $^1$H NMR spectrum consisted of two triplets at centered at 3.4 and 3.7 ppm and two quintets centered at 1.7 and 1.9 ppm. The triplets at 3.4 and 3.7 ppm are due to the CH$_2$ groups next to the Br (C4) and the OH (C1) group, respectively. The two quintets correspond to the two interior CH$_2$ groups on the alcohol (C2 & C3). Purity is noted from $^1$H NMR before measuring amounts for the next step (See Figures 2.3 and 2.4). For example, if the product is 50% pure, then twice as much was used for the next step.
**Figure 2.3** $^1$H NMR of ethyl-4-bromo-butyrate from the spectral database

**Figure 2.4** $^1$H NMR of 4-bromo-1-butanol after the LiAlH$_4$ Reduction (CDCl$_3$)

**PCC Oxidation**

Here the alcohol was converted into the aldehyde (BrCH$_2$CH$_2$CH$_2$CHO) and the chromium ion was reduced from Cr$^{6+}$ to Cr$^{3+}$. This oxidation has a 60-70% yield. The $^1$H NMR (Figure 2.5) revealed target peaks as a triplet centered
at 9.8 ppm for the proton attached to the carbonyl group (C1), a triplet centered at 3.4 ppm for the hydrogens at C4, a quartet centered at 2.7 ppm for the protons on C2, and a quintet centered at 2.2 ppm for the protons on C3. The triplet at 9.8 ppm may appear as a single peak because of its very low coupling constant.

Figure 2.5 $^1$H NMR of 4-bromo-1-butanal after the PCC oxidation (CDCl$_3$)

Condensation

In this step the aldehyde was connected to the pyrrole at the meso position to form a porphyrin ring consisting of four pyrroles and four propyl chains. This procedure was done in very low yield (0.6%). The C1 that was once an aldehyde group now forms the methine bridge of the porphyrin ring. The $^1$H NMR (Figure 2.6) yielded four peaks as confirmation of the porphyrin. There is one peak centered at 9.6 ppm corresponding to the presence of pyrrole, two triplets centered at 5.1 and 3.8 ppm corresponding to the substituent chain protons on C1 and C3 respectively, and one quintet centered at 3.1 ppm for the
protons on C2 of the substituent chain. The presence of porphyrin was also noted by its dark purple color. In solution the porphyrin gives off red fluorescence under long-wave UV light and it turns green in the presence of hydrochloric acid. The UV/Vis spectrum was also taken in chloroform to confirm the compound.

**Figure 2.6** $^1$H NMR after the Condensation step (CDCl$_3$)

The clay condensation improved the percent yield by more than 20 fold with a 12% yield (Table 2.1). The clay absorbed and removed many of the oxidants and polymeric by-products. The pores in the clay acted as a template for the formation of the bulky porphyrin molecule$^{20}$.

**Quaternization of Pyridine**

Here the bromine atom located at the end of each of the four substituent chains was replaced with pyridine where the nitrogen holds a positive charge. This reaction produces a water-soluble porphyrin with $^1$H NMR peaks as follows:
broad doublet centered at 9.2 ppm as pyrrole protons, a doublet at 9.1 ppm and triplets at 8.4 and 8.0 ppm that correspond to protons at the ortho, para, and meta positions on the pyridine respectively, a triplet at 5.2 ppm for protons on C1 and C3 of aliphatic substituent chain, and a quintet at 3.3 ppm for protons on C2 of the aliphatic chain. UV/Vis spectra were recorded in water and methanol.

Figure 2.7 shows the $^1$H NMR of the final pyridinium porphyrin salt.

**Figure 2.7 $^1$H NMR of the final product C3PyP (CD$_3$OD)**

**Inactivation Experiments**

The most effective porphyrins against *E. coli* were found to be those where the cation is at the end of a propyl, butyl, or pentyl, substituent chain (Table 2.2). Several inactivation times were examined for both *E. coli* and MS2. The data shown is for 30 minutes irradiation for *E. coli* and 1 minute for MS2.
These times were chosen based on the observance of equally extensive inactivation of *E. coli* and MS2 (~6 logs) as well as time constraints of the procedure. Since the porphyrins are more active in the light than in the dark, the MS2 dark samples are allowed 30 minutes rather than the 1 minute for irradiated samples to measure significant inactivation in the absence of light.

**Table 2.2** Porphyrin inactivation of undiluted *E. coli*

<table>
<thead>
<tr>
<th>Porphyrin</th>
<th>Log Reduction (Standard Deviation)</th>
<th>Light</th>
<th>Dark</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C3PyP</td>
<td></td>
<td>4.36 (0.38)</td>
<td>0.38 (0.09)</td>
</tr>
<tr>
<td>C4PyP</td>
<td></td>
<td>4.30 (0.22)</td>
<td>0.09 (1.17)</td>
</tr>
<tr>
<td>C5PyP</td>
<td></td>
<td>4.94 (0.05)</td>
<td>0.15 (0.06)</td>
</tr>
<tr>
<td>C7PyP</td>
<td></td>
<td>3.45 (0.26)</td>
<td>0.48 (0.13)</td>
</tr>
<tr>
<td>C11PyP</td>
<td></td>
<td>0.30 (0.05)</td>
<td>0.08 (0.003)</td>
</tr>
</tbody>
</table>

*Data were obtained from 30 min irradiation with a 40 W Hg fluorescent light (0.48 mW/cm²) 11.25” (measured by ruler) from wells with porphyrin and *E. coli* C3000. Experiments were done in triplicate with duplicate samples. There was no reduction in the concentration of *E. coli* samples exposed to light in the absence of porphyrin.

The *E. coli* inactivation was performed on two different *E. coli* mixtures. The original mixture (results shown in Table 2.3) was undiluted whereas in later experiments the mixture was diluted with 2:1 DIW to *E. coli* stock. From the original trials of the undiluted mixture, the C3PyP, C4PyP, and C5PyP exhibited efficient inactivation of *E. coli* while the C7PyP and C11PyP offered less inactivation. Based on their easier purification and greater inactivation, these three porphyrins and the commercially available meso-tetrakis(1-methy-4-pyridyl) 21H, 23H- porphyrine tetra-p- tosylate (TMPyP) were used in the *E. coli* and MS2
experiments that follow (See Figure 2.8). When the diluted E. coli mixture was used an increase in the inactivation was observed for the C3PyP and C4PyP, but the C5PYP decreased. See Table 2.3.

**Figure 2.8** The name and structure of porphyrins used in inactivation experiments with *E. coli* and MS2

- meso- tetrakis (3-[N-pyridiniumyl] propyl) porphyrin tetrabromide (C3PyP)

- meso- tetrakis (4-[N-pyridiniumyl] butyl) porphyrin tetrabromide (C4PyP)

- meso- tetrakis (5-[N-pyridiniumyl] pentyl) porphyrin tetrabromide (C5PyP)

- meso- tetrakis(1-methyl-4-pyridyl) 21H, 23H- porphyrine tetra-p- tosylate (TMPyP) commercially available
Table 2.3 1µM porphyrin reduction of diluted *E. coli* *

<table>
<thead>
<tr>
<th>Porphyrin</th>
<th>Light log reduction (Standard Deviation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3PyP</td>
<td>5.69 (1.27)</td>
</tr>
<tr>
<td>C4PyP</td>
<td>5.48 (0.37)</td>
</tr>
<tr>
<td>C5PyP</td>
<td>3.86 (0.68)</td>
</tr>
</tbody>
</table>

*The averages of three trials are shown. There was no observed dark inactivation in up to 30 min. *E. coli* solutions diluted 2:1, DIW: *E. Coli* was irradiated (40 W Hg fluorescent light, 0.48 mW/cm²) with 1 µM porphyrin for 30 min.

The porphyrins had a much more rapid inactivation of MS2 than the *E. coli*. With the most efficient of the porphyrins, nearly 6 logs reduction of the *E. coli* was achieved in 30 minutes (Table 2.3) where it took just one minute with the MS2. Without the irradiation, each of the porphyrins had less than one log reduction of both *E. coli* and MS2. The MS2 reductions are shown in Table 2.4.

Table 2.4 MS2 reductions with and without light*

<table>
<thead>
<tr>
<th>Porphyrin (1 µM)</th>
<th>Log reduction (Standard Deviation)</th>
<th>Light (1min)</th>
<th>Dark (30 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C3PyP</td>
<td></td>
<td>6.55 (0.71)</td>
<td>0.42 (0.17)</td>
</tr>
<tr>
<td>C4PyP</td>
<td></td>
<td>6.02 (0.19)</td>
<td>0.23 (0.08)</td>
</tr>
<tr>
<td>C5PyP</td>
<td></td>
<td>4.08 (1.14)</td>
<td>0.22 (0.14)</td>
</tr>
</tbody>
</table>

*MS2 was irradiated with a 40 W Hg fluorescent light, 0.48 mW/cm². Data shown is the average of three trials.

Additional experiments were done using the commercially available porphyrin TMPyP against the bacteriophage MS2. This was done to compare the synthetic porphyrins inactivation to a similar compound with constrained positive charges. This porphyrin was measured at three concentrations: 0.1, 1.0, and 10 µM. For each concentration, MS2 was measured at 1 minute of light contact time, as well
as at 1 and 30 minutes of contact time in the absence of light. Table 2.6 shows the results for the TMPyP experiments.

**Table 2.5** MS2 reductions using the commercially available TMPyP

<table>
<thead>
<tr>
<th>Concentration of TMPyP (µM)</th>
<th>Log reduction (Standard Deviation)</th>
<th>Light (1min)</th>
<th>Dark (30min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td></td>
<td>6.29 (0.02)</td>
<td>1.45 (0.10)</td>
</tr>
<tr>
<td>1.0</td>
<td>&lt; 9.17*</td>
<td>6.14 (0.27)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>&lt; 9.17*</td>
<td>7.49 (0.95)</td>
<td></td>
</tr>
</tbody>
</table>

*This data is below the limit of detection. *MS2 was irradiated with a 40 W Hg fluorescent light, 0.48 mW/cm². Data shown is the average of three trials.

**Discussion**

A total of five porphyrins were synthesized and tested against *E. coli*, and the three most efficient porphyrins, C3PyP, C4PyP, and C5PyP, were more extensively tested against bacteriophage MS2. Each of the porphyrins synthesized has four identical substituents at the meso position of the porphine ring. This four-fold symmetry allows for a more straightforward procedure than would a synthesis of porphyrins with varying substituents. All data collected showed that the synthesis was successful in producing five distinct porphyrins. In each step of the synthesis, reactions seemed to have greater yield as the reaction mixtures were more dilute. In the condensation procedure, the use of the montmorillonite clay as a catalyst was much more effective than the method involving trifluoroacetic acid.

For the inactivation experiments, large concentrations of both the bacteria and bacteriophage were needed in order to extensively measure inactivation by
the porphyrins. The extent of inactivation and the ability to measure inactivation as a function of time was dependent on the rate of inactivation of each microbe, the concentration of microbes in solution, and the concentration of porphyrin used. A measurement of inactivation as a function of time was not feasible for MS2, which showed greater than six-logs reduction (99.9999%) with just one minute of light exposure. At five minutes the MS2 was reduced to below the limit of detection. Although the target concentration of porphyrin was 1 µM (~1 mg/L), which is comparable to drinking water limits on chlorine, inactivation was observed at 0.1 and 10 µM to ensure data within the limits of detection. The 1 µM porphyrin concentration is also appealing because at this concentration there was a high level of inactivation of *E. coli* with light with no significant dark toxicity.

The bacteria and all host cells used must be in the log phase of growth. If non-viable bacteria compete for contact with the porphyrin, then the porphyrin will appear to be less efficient against the viable bacteria. While contaminated waters in the environment would not contain bacteria in log phase growth, higher concentrations of bacteria are needed for a proof of concept for porphyrin use in PDI.

The amount of turbidity in solution also had an effect on the porphyrin’s efficiency as a disinfectant. The *E. coli* solution used to obtain the primary data in table 2.2 was undiluted, while that for the data in table 2.3 was diluted with DIW. This dilution reduced the amount of suspended solids that can block light and compete for porphyrin contact, which lead to an increase in the overall inactivation of *E. coli*. 
Turbidity, a measurement of cloudiness, was measured for both solutions of microbes. This will give some information as to the stages of water treatment at which the porphyrin will be most effective and allow for comparison of the porphyrin data to that of other disinfectants used in water treatment processes. The most common disinfectants for drinking water are generally used as the last step in water treatment. This occurs after most of the turbidity is removed from the water. It is assumed that the lower the turbidity, the higher the porphyrin’s efficiency of inactivating the test microbes.

When comparing the newly synthesized porphyrins to literature on free chlorine\textsuperscript{21}, the most widely used disinfectant for water treatment\textsuperscript{1}, the flexible cation porphyrins are potentially more powerful disinfectants. Tables 2.6 and 2.7 show the comparisons of the porphyrins’ and free chlorine inactivation of \textit{E. coli} and MS2 respectively.
Table 2.6 Comparisons of C3PyP and free chlorine inactivation of *E. coli*

<table>
<thead>
<tr>
<th>System Characteristics</th>
<th>Free Chlorine (^{21})</th>
<th>C3PyP (present study)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microbe</td>
<td><em>E. coli</em></td>
<td><em>E. coli</em></td>
</tr>
<tr>
<td>Disinfectant Concentration</td>
<td>100 mg/L</td>
<td>1 mg/L</td>
</tr>
<tr>
<td>Temperature ºC</td>
<td>25ºC</td>
<td>25ºC</td>
</tr>
<tr>
<td>Nephelometric Turbidity Units (NTU)</td>
<td>200 NTU</td>
<td>132 NTU</td>
</tr>
<tr>
<td>Log Reduction (approx)</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Contact Time min</td>
<td>2</td>
<td>30</td>
</tr>
<tr>
<td>CT constant mg·min/L</td>
<td>200</td>
<td>30</td>
</tr>
</tbody>
</table>

Table 2.7 Comparisons of C3PyP and free chlorine inactivation of MS2.

<table>
<thead>
<tr>
<th>System Characteristics</th>
<th>Free Chlorine (^{21})</th>
<th>C3PyP (present study)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microbe</td>
<td>MS2</td>
<td>MS2</td>
</tr>
<tr>
<td>Disinfectant Concentration</td>
<td>100 mg/L</td>
<td>1 mg/L</td>
</tr>
<tr>
<td>Temperature ºC</td>
<td>25ºC</td>
<td>25ºC</td>
</tr>
<tr>
<td>Nephelometric Turbidity Units (NTU)</td>
<td>2 NTU</td>
<td>0.23 NTU</td>
</tr>
<tr>
<td>Log Reduction (approx)</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Contact Time min</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>CT constant mg·min/L</td>
<td>200</td>
<td>1</td>
</tr>
</tbody>
</table>

Although the two studies were not done under the same conditions, the tests are similar enough to compare CT constant, which is the product of the...
concentration (mg/L) of the disinfectant and the time needed (min) for a specific level of inactivation. This value expresses a specific level of inactivation as a function of disinfectant concentration multiplied by contact time. Tables 2.6 and 2.7 show that the most efficient porphyrin offered more inactivation of the test microbes with a much smaller CT constant. These preliminary studies have shown that these porphyrin compounds could be a more effective disinfectant than free chlorine, especially in turbid waters.

With the comparison of TMPyP to our synthetic porphyrins in the inactivation of MS2, one can see that the TMPyP is much more lethal to MS2 than the synthetic porphyrins. This is true both in the presence and absence of light. The increase in the MS2 inactivation in the absence of light is due to an increase in natural toxicity of the porphyrin (toxicity in the absence of light). This shows that the TMPyP is not as inert in the absence of light as the other porphyrins tested, and could potentially have more adverse effects if ingested. This could very well be due to the different counter ions in the commercially available TMPyP and our synthetic porphyrins. The synthetic porphyrins have a simple bromine counterion where as the TMPyP has a more complex, and potentially more toxic, tosylate counter ion. In order to directly measure the effect of cation position, a series of fixed-cation porphyrins with identical counterions were synthesized. These porphyrin analogs have the same molecular formula as our newly synthesized porphyrins, with the location of the cation being the only difference in structure. This direct comparison was needed
to help to better understand the porphyrins’ mechanism of inactivation and the role of this series of porphyrins as potential disinfectants of the future.
References


Chapter 3: Porphyrin Photodegradation and Toxicity

Background

Photodynamic inactivation (PDI) of pathogens is an effective method of disinfection that has not been applied to water treatment. In PDI, a chromophore absorbs energy from light and ultimately transfers the energy to dioxygen to generate the singlet oxygen that inactivates pathogens. Additionally, PDI is an appealing method of disinfection because its mechanism of action is not likely to lead to resistance, and if the photosensitizer (PS) is ingested, its toxicity is reduced in the absence of light. The principles of PDI are evident in photodynamic therapy (PDT), an approved treatment for several cancers\(^1\)-\(^5\), and they have more recently been applied to the disinfection of blood products\(^6\)-\(^10\).

Cationic porphyrins are one class of chromophores that have proven to be effective in the inactivation of viral, bacterial, fungal and parasitic pathogens\(^2,4,11,16-18,20,23\). In addition to being highly efficient PSs, the physicochemical properties of porphyrins may readily be tuned by altering the peripheral substituents. Recent studies have shown that cationic porphyrins irradiated with light are particularly efficient in the inactivation of non-enveloped viruses as well as gram-negative bacteria in aqueous media\(^10\)-\(^19\). It is believed that the cationic character affords better association with the predominantly negatively charged surfaces of non-enveloped viruses and gram-negative bacteria. Furthermore, most pathogens of concern in modern drinking water treatment, have an overall
negatively charged surface\textsuperscript{24}. To date, several studies have shown some connection between the cationic character of the PS and the association of that PS to gram-negative bacteria\textsuperscript{11, 18, 20, 21}.

The cationic porphyrins used in previous tests have rigidly attached positive charges on the periphery of the tetrapyrrole macrocycle. In the present study, it is hypothesized that attaching cationic groups via more flexible, aliphatic links will allow for increased cation mobility and better adaptation to the shape and negative charge distribution on the surface of target microorganisms. To evaluate this hypothesis we have synthesized porphyrin analogs to C3PyP and C4PyP, the most efficient porphyrins from the PDI experiments summarized in Chapter 2. These porphyrin analogs have the same molecular formula and differ only in the cation position and flexibility. The comparison of these porphyrins will allow for a direct observation of the effect of the cation position on various properties that effect PDI.

To address the second and third specific aims of this research, the porphyrins’ stability and toxicity in the presence and absence of light was examined. A better understanding of porphyrin stability with and without activation is critical for the practical application of these porphyrins and PDI in general. A photostability test based on previous literature\textsuperscript{17} was used to monitor the degradation of each of the porphyrins. In this study, the photostability of four porphyrins that differ only in the mobility of the cation was investigated. The formation of photoproducts was monitored, and an attempt was made to define the degradation products of these porphyrins using \textsuperscript{1}H NMR, UV/Vis and mass
spectrometry. The toxicity of the parent porphyrins and the photoproducts to model mammalian cells was measured in order to understand the potential of adverse effects of these porphyrins. The porphyrins incorporated into this study can be seen in Figure 3.1.

**Figure 3.1** The four porphyrins used in this study and their abbreviations

- **Meso-tetrakis (3-[N-pyridiniumyl] propyl) porphyrin tetrabromide (C3PyP)**
- **Meso-tetrakis (4-[N-pyridiniumyl] propyl) porphyrin tetrabromide (C4PyP)**
- **Meso-tetrakis (3-[N-propyl] pyridiniumyl) porphyrin tetrabromide (TProPyP)**
- **Meso-tetrakis (4-[N-butyl] pyridiniumyl) porphyrin tetrabromide (TBuPyP)**
**Materials and Methods**

*Porphyrin synthesis*

The C3PyP and C4PyP porphyrins were synthesized in our laboratory as described in Chapter 2. TBuPyP and TProPyP were synthesized from tetra *meso* pyridinium porphyrins (Frontier Scientific, Logan UT), which were refluxed with either 1-bromopropane or 1-bromobutane respectively to obtain the TProPyP and TBuPyP. All porphyrins were purified by serial extractions from aqueous solution with chloroform, methanol, ether and water followed by lyophilization for 48 hours. All other materials were obtained through Fisher Scientific.

*Porphyrin Photodegradation and Photoproduct Formation*

A stirred solution of 5 mL of 1 mM porphyrin in DIW in a 20 mL glass vial was irradiated with 300 W halogen projector (4.72 mW/cm²) for 24 hours. Aliquots (200 µL) were taken at time zero and once every hour for 6 hours and at 24 hours. To compensate for evaporation, the reaction vial was weighed at t=0, and before and after the removal of each aliquot (Sample calculations can be seen in the Appendix 3). Each aliquot was frozen, dried by lyophilizer and analyzed by ¹H NMR and MS. At each sample time 10 µL of the test solution was diluted with 3% sodium dodecyl sulfate (SDS) in DIW to 10 µM, vortexed, and analyzed by UV/VIs. Separately, the oxidation was observed under anoxic conditions by initially bubbling argon through the test solution for 30 minutes and maintaining positive argon pressure during the irradiation.
Porphyrin degradation was measured as the decrease in the Soret band at 416 nm (C3PyP and C4PyP) or 432 nm (TProPyP and TBuPyP). The UV/Vis (Varian Cary 300) was scanned from 190-900 nm at 350 nm/min. After 24 hours of irradiation, regardless if the Soret band had an appreciable decrease, the porphyrin and soluble photoproducts were transferred to sterile vials and stored at 4°C until they were later used in the toxicity study.

Toxicity to Mammalian Chinese Hamster Ovary (CHO) cells

CHO cells were obtained from the American Type Culture Collection (ATCC) and maintained according to their specifications. All porphyrin photoproducts in the following toxicity assay were irradiated for 24 hrs. Various concentrations of the parent and degraded porphyrin were added to 4 mL of CHO cells (~220 cells/well) in PBS buffer (pH 7) in a six-well plate. The plates were shielded from light and incubated at 37°C with 5% CO₂ for one week. The cells were then fixed with a 1:1 mixture of ethanol and glacial acetic acid, stained with crystal violet, and the visible cells were counted.

Results

The original porphyrin is a dark purple color, while after several hours of light irradiation the color is orange. For the photostability tests, the porphyrins degraded faster under light than without the irradiation. Porphyrins in solution, maintained in sealed vials and shielded from light at 4°C, were found to show no decrease in the Soret band for periods exceeding one month (data not shown). The TProPyP and TBuPyP were much more stable than the flexible cation analogs. After 24 hours of exposure to visible light, the C3PyP and C4PyP
completely degraded (no peak at 416 nm). An example of the C4PyP UV/Vis spectrum can be seen in Figure 3.2. The TProPyP and TBuPyP porphyrins maintained their maximum band and color beyond 24 hours of irradiation. See Figure 3.3.

**Figure 3.2** The degradation of C4PyP over time*

*Measurements were taken at various times of irradiation up to 24 hrs and diluted to 10 µM prior to analysis. All of the porphyrins are marked by four additional peaks (Q bands) to the right of the Soret band. These additional signature peaks also degraded with irradiation time while the peaks to the left of the Soret for all porphyrins seemed to increase slightly with increased irradiation. The bottom spectrum is an expansion of the top spectrum in which the absorbance of the Soret is reduced by the time (hours) of exposure to light; in descending order of peak height, t=0 (red), t=1 (purple), t=2 (light green), t=3 (brown), t=4 (green), and t=24 (black)
Figure 3.3 The absorbance of the porphyrin Soret over time*

*Measurements were taken at various times of irradiation up to 24 hrs and diluted to 10 µM prior to analysis.

From the $^1$H NMR and MS, it is evident that the C3PyP and C4PyP porphyrin, open to the atmosphere, have completely degraded under light and new photoproducts were formed. The TProPyP and TBuPyP showed little change in the mass spectrum or $^1$H NMR after 24 hours of light. The $^1$H NMR of the TProPyP and TBuPyP before and after irradiation can be seen in Appendix 2.

After 24 hours of irradiation, both the $^1$H NMR (Figures 3.4 and 3.5) and MS (Table 3.1) of the C3PyP and C4PyP yielded very different spectra from that of the parent compound.
Figure 3.4 The $^1$H NMR of C3PYP before (A) and after (B) 24 hours of irradiation ($D_2O$)
Figure 3.5 The $^1$H NMR of C4PYP before (A) and after (B) 24 hours of irradiation ($D_2O$)

A.

B.
**Table 3.1** The MS peaks of the four porphyrins and their photoproducts*

<table>
<thead>
<tr>
<th>Porphyrins</th>
<th>Major Peaks M/z (Relative Abundance)</th>
<th>Peak Explanations</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3PyP MW 1110.6</td>
<td>237.87 (100%)</td>
<td>Mass $4^+$-2Br</td>
</tr>
<tr>
<td></td>
<td>263.13 (100%)</td>
<td>Mass $3^+$-4Br</td>
</tr>
<tr>
<td>C3 Photoproduct</td>
<td>181.66 (100%)</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>379.10 (98%)</td>
<td>N/A</td>
</tr>
<tr>
<td>C4PyP MW 1166.7</td>
<td>211.73 (100%)</td>
<td>Mass $4^+$-4Br</td>
</tr>
<tr>
<td></td>
<td>309.13 (100%)</td>
<td>Mass $3^+$-3Br</td>
</tr>
<tr>
<td>C4 Photoproduct</td>
<td>195.6 (100%)</td>
<td>N/A</td>
</tr>
<tr>
<td>TProPyP</td>
<td>263.27 (100%)</td>
<td>Mass $3^+$-4Br</td>
</tr>
<tr>
<td></td>
<td>394.33 (80%)</td>
<td>Mass $2^+$-4Br</td>
</tr>
<tr>
<td></td>
<td>787.40 (80%)</td>
<td>Mass $1^+$-4Br</td>
</tr>
<tr>
<td>TPro Photoproduct</td>
<td>Same as parent</td>
<td>Same as parent</td>
</tr>
<tr>
<td>TBuPyP</td>
<td>281.93 (100%)</td>
<td>Mass $3^+$-4Br</td>
</tr>
<tr>
<td></td>
<td>394.27 (100%)</td>
<td>Mass $2^+$-4Br-4(CH2)</td>
</tr>
<tr>
<td></td>
<td>731.40 (80%)</td>
<td>Mass $1^+$-4Br-8(CH2)</td>
</tr>
<tr>
<td>TBu Photoproduct</td>
<td>Same as parent</td>
<td>Same as parent</td>
</tr>
</tbody>
</table>

*The TProPyP and TBuPyP showed no change in the mass spectrum after 24hrs of light. The C3 and C4 photoproducts could not be determined using MS by the present methods.*
Reducing the oxygen in the system reduced the porphyrins’ degradation. Under anoxic conditions, the C3PyP and C4PyP retained greater than 70% and 80% of their Soret band respectively in the same time that oxygenated samples were completely degraded (no Soret Band). When comparing the partially and fully degraded samples, both methods gave rise to the same photoproducts. In the anoxic system, trace levels of oxygen may have influenced some photoproduct formation. The fact that the porphyrin degradation is inhibited by anoxic conditions suggests that oxygen plays a role in the photodegradation of the porphyrins.

While the TProPyP and TBuPyP showed no measurable degradation, the toxicity of each of the porphyrin/photoproduct mixtures was measured. A linear relationship was found between porphyrin concentration and percent survival, and the concentration needed to kill 50% of the cells (LC50) was calculated (See Table 3.2).
Table 3.2 The LC50s for each of the porphyrins and their photoproducts\textsuperscript{a}

<table>
<thead>
<tr>
<th>Porphyrin</th>
<th>Average LC50 (µM) (Standard deviation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3PyP</td>
<td>&gt;500\textsuperscript{b}</td>
</tr>
<tr>
<td>C3Product</td>
<td>6.5 (0.19)</td>
</tr>
<tr>
<td>C4PyP</td>
<td>&gt;500\textsuperscript{b}</td>
</tr>
<tr>
<td>C4Product</td>
<td>7.5 (0.01)</td>
</tr>
<tr>
<td>TProPyP</td>
<td>14.6 (0.6)</td>
</tr>
<tr>
<td>TBuPyP</td>
<td>25.1 (5.9)</td>
</tr>
</tbody>
</table>

\textsuperscript{a}The concentration of porphyrins and photoproducts needed to kill 50% of Chinese Hamster Ovary (CHO) cells is shown. This is an average of three separate trials, each done in duplicate. The TProPyP and TBuPyP showed no product formation, and the toxicity of irradiated samples was within the stdev of the original samples.

\textsuperscript{b}These values are of the highest concentration measured, in which both C3PyP and C4PyP were found to be nontoxic.

Discussion

Photostability and photoproduct formation

The C3PyP and C4PyP porphyrins degraded much faster than their fixed-cation analogs. This is likely due to the pyridinium at the meso position of the analogs which can block nucleophilic attack at the most vulnerable position. Also the pyridine ring at the meso position of the porphyrins allows for increased aromaticity, which could also contribute to its stability. Even still, the C3PyP and C4PyP porphyrins took longer than 4 hours to completely degrade. This is more than several times the period required for reasonable inactivation of microorganisms\textsuperscript{17, 18, 20}. 
While the C3PyP and C4PyP products were not clearly defined by the MS, by monitoring the $^1$H NMR over various lengths of irradiation it was evident that the pyridine is first to fragment (Figures 3.3 and 3.4 A, 7-9 ppm), followed by the break in the porphyrin macrocycle (Figures 3.3 and 3.4 A. 9.8 ppm). Previous literature and the present data suggest that the photoproducts consist of oxidized pyrrole and dipyrrole derivatives, which are likely accompanied by the 3 and 4 carbon chains, and some type of pyridinium salt\textsuperscript{25}.

There are two types of porphyrin photodegradation\textsuperscript{26}: one in which the chromophore remains intact in some modified state, and the second in which the compound is broken into smaller fragments that no longer absorb light in the visible region. While the TBuPyP and TProPyP may have shown some degradation, the fact that they maintained their Soret band over 24 hours of irradiation may suggest a photoproduct with intact chromophore; however, the C3PyP and C4PyP photoproducts are definitely fragmented.

**Toxicity**

With the four porphyrins examined, some general trends were observed. The C3PyP and C4PyP were less toxic in their native state (prior to irradiation) than their fixed-cation analogs, but had more toxic photoproducts. The TProPyP and TBuPyP, which both showed little degradation over 24 hours, showed little difference in toxicity between the parent compounds and the irradiated photoproducts. While the C3PyP and C4PyP and their photoproducts were nontoxic at the levels suggested for effective disinfection (1 µM), the fact that the photoproducts are more toxic is of some concern. The data suggest that the
photoproducts of these porphyrins could be tens of times more toxic than the parent compounds and should be used to assess environmentally safe concentrations. If these products were to accumulate in the drinking water system or in the body, they could have adverse effects.
References


11 M. Merchat, G. Bertolini, P. Giacomini, A. Villaneuva and G. Jori, Meso-substituted cationic porphyrins as efficient photosensitizers of gram-positive and


Chapter 4: Porphyrin Binding, ROS production, and Subsequent Inactivation

Background

Recent studies have shown that cationic photosensitizers (PSs), irradiated with light, are particularly efficient in the inactivation of non-enveloped viruses as well as gram-negative bacteria in aqueous media 1-10. It is believed that the positive charge on cationic PSs help them to better associate with the predominantly negatively charged surfaces on target pathogens. Since the diffusion distance of singlet oxygen, the reactive oxygen species involved in PDI, is roughly 50 nm 11, bringing the PS in close proximity to the target is imperative for the short-lived ROS to reach and damage the target pathogen.

To date several studies have shown some connection between cationic character of the PS and the binding of that PS to gram-negative bacteria 1, 9, 10, 12. However, in some studies using various cationic PSs, the PS uptake by the cell does not fully explain levels of inactivation 1, 9, 12. Some PSs that have a greater overall uptake still result in less overall inactivation. The ultimate location of the PS bound to or incorporated into the cell may also play a large role in the efficiency of PDI. PDI from porphyrin association with the outer surface of bacteria and viruses could be different from its actual uptake and incorporation into the bacterial cell. If the inactivation is driven by damage to the pathogen’s genetic material, then porphyrin uptake is instrumental to PDI; however, if
oxidation of the pathogen surface (outer membrane, cell wall, capsid, envelope, etc.) is the predominate mechanism for PDI, then uptake may be less important. Furthermore, PS uptake could feasibly be counterproductive to PDI if the PS is altered in the uptake process, shielded from light within the cell, or removed from the sites on or near the outer surface that are critical to PDI. While the rapid inactivation of MS2 observed in studies reported in Chapter 2 (nearly 6 logs reduction in 1 min) would suggest surface interactions to be the dominant mode of action, it is possible that different types of pathogens (bacteria, viruses, parasites) will undergo different routes of inactivation by photodynamic processes.

Cationic porphyrins are one group of chromophores that have proven to be effective in the inactivation of viral, bacterial, and fungal and parasitic pathogens.\textsuperscript{1, 7-10, 13-15} The cationic porphyrins used in previous tests have carried fixed positive charges on the periphery of the tetrapyrrole macrocycle. Porphyrins with positive charges attached farther from the ring through an aliphatic carbon chain have been synthesized to allow the positive charge to move more freely and possibly better adapt to the shape and negative charge distribution on the surface of target microorganisms. Porphyrin analogs with the positive charge connected at the periphery of the porphyrin macrocycle were also synthesized to observe the effects of positive charge position on the binding to and subsequent inactivation of \textit{E. coli} and \textit{Salmonella LT2} and their corresponding bacteriophages. The singlet oxygen production was also observed, and an attempt was made to distinguish the final localization of
porphyrin associated with the target cells. The porphyrins used in this study can be seen in Figure 4.1.

**Figure 4.1** The four porphyrins used in this study and their abbreviations

\[\text{Meso-tetrakis (3-[N-pyridiniumyl] propyl) porphyrin tetrabromide}\]

\[\text{Meso-tetrakis (4-[N-pyridiniumyl] butyl) porphyrin tetrabromide}\]

\[\text{Meso-tetrakis (3-[N-propyl] pyridiniumyl) porphyrin tetrabromide}\]

\[\text{Meso-tetrakis (4-[N-butyl] pyridiniumyl) porphyrin tetrabromide}\]

**Materials and Methods**

*Porphyrins*

All porphyrins were synthesized in our laboratory as previously described in chapter 3.
Microbial Inactivation

*E. coli FAmp* (ATCC 700891), *Salmonella LT2* (ATCC19585), MS2 (ATCC19585), and PDR-1 (ATCC 700044) were used in the following experiments. The bacterial host, *Salmonella LT2* or *E. coli FAmp*, was inoculated into 30 mL of TSB and incubated for 18 hours at 37°C in a shaking incubator prior to experimentation. 100 µL of the sample was seeded into 30 mL of TSB and incubated for 4 more hours on the day of the experiment. The 4-hour stock was diluted 2 parts PBS to one part stock and used in the bacterial inactivation and binding experiments (10^8-10^9 cfu/mL). Undiluted stock was used as a host for the bacteriophage inactivation. 5 mL of this dilution was placed into a vial with a stir bar and 5 µL of 1mM porphyrin to make a final concentration of 1 µM. At specific intervals of irradiation with a 300W halogen projector (4.72 mW/cm²), starting from T=0, a 10 µL sample of the solution was removed, diluted to a countable range, and transferred onto Petri plates of TSA using top agar (TSB with 0.07% Agar); 3 dilutions were plated for each time period. The agar was allowed time to cool and gel; plates were inverted and incubated at 37°C with 5% CO₂ overnight. Colonies appear as opaque dots upon a clear surface; bacterial concentrations were measured as colony forming units (cfu) per mL.

Controls included samples with porphyrins and test microbes without irradiation, top and bottom agar only, and the microbe with irradiation and no porphyrin. Test concentrations were compared to those of samples from the same bacterial stock before the addition of porphyrin and light to calculate the log reduction of bacteria.
For bacteriophage inactivation, 600 µl of phage stock ($10^{11}$ and $10^{13}$ cfu/mL for MS2 and PRD-1 respectively) was added to 12 mL of PBS and exposed to the porphyrin and light as described above. This same stock is used in later binding experiments. After 1 min (MS2) and 30 min (PRD-1), the samples were diluted and plated with 150 µl of the bacterial host (E. coli for MS2 and Salmonella for PRD-1). Plaques, which originate from a single virus particle’s infection and rupture of a host cell, appear as a clear patch on an opaque confluent lawn. Phage concentrations were measured as plaque forming units (pfu) per mL.

**Porphyrin Binding**

This procedure was adapted from a combination of photosensitizer-bacteria binding studies from previous literature $^1$, $^9$, $^{10}$. The Salmonella and E. coli stocks described above were quantified using the described pour-plate method. 5 µL of each porphyrin (1 mM) was separately added to 5 mL of the diluted bacterial solution to achieve 1 µM porphyrin. After allowing 5 min for efficient binding$^9$, four 1 ml aliquots were then placed in 1.5 mL centrifuge tubes so that there were four samples for each porphyrin/bacterium combination. All of the samples were then centrifuged at 13,000 g for 5 minutes. The supernatant was removed, and the cells were resuspended by vortexing in 1 mL PBS. The porphyrin washing was repeated three times in total. After the third supernatant was removed, the cells were resuspended in 3% sodium dodecyl sulfate (SDS; 3 g/100 mL DIW). The fluorescence of two of the samples for each porphyrin was
measured immediately, while the other two samples were placed in a sonicator for 30 min at room temperature prior to the measurement of fluorescence.

The binding after sonication was meant to assess the amount of porphyrin uptake into the cell. The series of porphyrin washings should remove the porphyrins that are not at least loosely bound to the bacteria. The 30-minute sonication should induce cell lysis and release any porphyrins within the cell.

To measure porphyrin binding to bacteriophages, a similar method was used with the previously-described bacteriophage stock. An ultra-centrifuge equipped with a swinging bucket rotor was used to pellet the virus at 35,000 g for 3 hours, and the supernatant was removed. The pellet was resuspended in 5 mL of PBS and compared to a 0.25 µM porphyrin sample. In order to account for all of the porphyrin in solution, the fluorescence of both the supernatant and pellet resuspension were analyzed as described above and reported as the percent of the porphyrin remaining when compared to the original 1 µM concentration.

Fluorescence Measurement

All porphyrins in this study yield a maximum emission around 655-660 nm. The settings on the spectrofluorimeter (Perkin-Elmer model 650-10S) were as follows: PM Gain, Mode and Response were all set to Norm, Scan Speed was 60 nm/min, recorder speed was 0.5 cm/min. The peak height of each sample was compared to calibration curves of the pure porphyrins in 3% SDS using the relative intensity of the 0.25 µM concentration as a reference point. The C3PyP and C4PyP porphyrins were excited at 416 nm, while the TBuPyP and TProPyP were excited at 432 nm. The emission of red fluorescence was recorded from
Prior to measuring the fluorescence of test samples, a 1µM and 0.25 µM porphyrin sample were recorded. The 1 µM sample should yield a reading of 90-110% fluorescence (the full length of the graph). The same spectrofluorimeter settings were used to measure the 0.25 µM standard and the four test samples for that specific porphyrin.

**Singlet Oxygen Production**

Singlet oxygen production was measured by the reaction with furfuryl alcohol (FFA) that forms one major product that can be analyzed by high performance liquid chromatography (HPLC)\(^\text{16}\). A 5 mL solution of porphyrin (10 µM) and FFA (10 µM) in PBS was irradiated using above-described light for 2 hours. A 1.5 mL sample was taken, and phenyl 1-1, 2-ethanediol was added as an internal standard (10 µM). The samples were separated and analyzed on a Shimadzu HPLC/ UV (LC2010A HT) with RP column (Zorbax, 4.5 mm x 25 cm, P.N. 880952.702) with a flow rate of 0.4 mL per min, 70% DIW 30% methanol. The samples were quantified by UV/Vis at 219 nm, and the area under the curve was integrated using computer software (Shimadzu EZ start version 7.3 SP1, Build 13).

**Results**

Tables 4.1 and 4.2 show the porphyrins’ inactivation of *E. coli* and *Salmonella*, respectively. The controls (bacteria with light irradiation in the absence of porphyrin, and bacteria and porphyrins in the absence of light) yielded concentrations within the 95% confidence intervals of the original test bacterial stock (data not shown), which indicates that there is little or no
inactivation due to the light or porphyrin alone. The 60 minute dark time is a rough estimate and may vary due to the time required to plate all light samples prior to plating dark samples.

**Table 4.1** Inactivation of *E. coli* by fixed and flexible cationic porphyrins

<table>
<thead>
<tr>
<th>Porphyrin (1µM)</th>
<th>Log reduction (Standard Deviation)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Light 30 min</td>
<td>Dark 60 min</td>
<td></td>
</tr>
<tr>
<td>C3PyP</td>
<td>2.22 (0.16)</td>
<td>-0.09 (0.13)</td>
<td></td>
</tr>
<tr>
<td>C4PyP</td>
<td>3.45 (1.22)</td>
<td>-0.001 (0.12)</td>
<td></td>
</tr>
<tr>
<td>TProPyP</td>
<td>0.06 (0.09)</td>
<td>-0.17 (0.03)</td>
<td></td>
</tr>
<tr>
<td>TBuPyP</td>
<td>1.75 (1.36)</td>
<td>0.023 (0.08)</td>
<td></td>
</tr>
</tbody>
</table>

*These data were obtained from three trials, each using duplicate samples. Standard deviation is given in parenthesis. The negative log reduction actually shows an increase in the concentration of bacteria.

**Table 4.2** Inactivation of *Salmonella* by fixed and flexible cationic porphyrins

<table>
<thead>
<tr>
<th>Porphyrin (1µM)</th>
<th>Log reduction (Standard Deviation)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Light 30 min</td>
<td>Dark 60 min</td>
<td></td>
</tr>
<tr>
<td>C3PyP</td>
<td>4.08 (0.47)</td>
<td>-0.12 (0.05)</td>
<td></td>
</tr>
<tr>
<td>C4PyP</td>
<td>4.37 (0.35)</td>
<td>-0.06 (0.06)</td>
<td></td>
</tr>
<tr>
<td>TProPyP</td>
<td>0.09 (0.07)</td>
<td>-0.18 (0.07)</td>
<td></td>
</tr>
<tr>
<td>TBuPyP</td>
<td>1.42 (1.84)</td>
<td>0.13 (0.13)</td>
<td></td>
</tr>
</tbody>
</table>

*These data were obtained from three trials, each using duplicate samples. Standard deviation is given in parenthesis. The negative log reduction actually shows an increase in the concentration of bacteria.

Tables 4.3 and 4.4 show the porphyrins' binding to *E. coli* and *Salmonella*. From the average concentrations of porphyrin and bacteria from the inactivation experiments, the concentration of porphyrin per $10^8$ colony forming units (CFU) was calculated. The TProPyP binding to both *E. coli* and *Salmonella* was below the limit of detection using the pre-described method.
### Table 4.3 Porphyrin binding to *E. coli*

<table>
<thead>
<tr>
<th>Porphyrin</th>
<th>nmol/10⁸ CFU</th>
<th>95% Upper-lower limit</th>
<th>Sonicated nmol/10⁸ CFU</th>
<th>95% Upper-lower limit</th>
<th>Difference in sonication nmol/CFU</th>
<th>95% Upper-lower limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3PyP</td>
<td>0.93</td>
<td>0.57-1.30</td>
<td>0.92</td>
<td>0.50-1.33</td>
<td>-0.02</td>
<td>-0.09-0.06</td>
</tr>
<tr>
<td>C4PyP</td>
<td>1.33</td>
<td>0.24-2.43</td>
<td>1.39</td>
<td>-0.11-2.88</td>
<td>0.05</td>
<td>-0.41-0.51</td>
</tr>
<tr>
<td>TProPyP</td>
<td>&lt;MDL</td>
<td>-</td>
<td>&lt;MDL</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TBuPyP</td>
<td>0.89</td>
<td>0.33-1.46</td>
<td>1.11</td>
<td>0.62-1.59</td>
<td>0.21</td>
<td>0.06-0.37</td>
</tr>
</tbody>
</table>

*(<MDL is below the method detection limit, which was 2.24 nM for TProPyP, which corresponds to 0.448-0.224 pmol/10⁸CFU) Porphyrins and bacteria were centrifuged at 13000 g for 5 min, washed three times with PBS, analyzed on spectrofluorimeter and compared to a standard curve. Like samples were sonicated for 30 min to separate the membrane-bound porphyrin with that incorporated into the cell. Data shown are the average of three trials, each done in duplicate. The upper and lower limit that encompasses 95% of the data is shown on the left of the corresponding column.*

### Table 4.4 Porphyrin binding to *Salmonella LT2*

<table>
<thead>
<tr>
<th>Porphyrin</th>
<th>nmol/10⁸ CFU</th>
<th>95% Upper-lower limit</th>
<th>Sonicated nmol/10⁸ CFU</th>
<th>95% Upper-lower limit</th>
<th>Difference in sonication nmol/10⁸CFU</th>
<th>95% Upper-lower limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3PyP</td>
<td>2.14</td>
<td>1.71-2.57</td>
<td>2.54</td>
<td>2.03-3.04</td>
<td>0.40</td>
<td>-0.30-1.09</td>
</tr>
<tr>
<td>C4PyP</td>
<td>1.70</td>
<td>0.98-2.41</td>
<td>2.32</td>
<td>1.19-3.44</td>
<td>0.62</td>
<td>0.19-1.05</td>
</tr>
<tr>
<td>TProPyP</td>
<td>&lt;MDL</td>
<td>-</td>
<td>&lt;MDL</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TBuPyP</td>
<td>2.118</td>
<td>0.25-4.10</td>
<td>2.70</td>
<td>0.14-5.27</td>
<td>0.53</td>
<td>-0.12-1.17</td>
</tr>
</tbody>
</table>

*(<MDL is below the method detection limit, which was 2.24 nM for TProPyP, which corresponds to 0.448-0.224 pmol/10⁸CFU) Porphyrins and bacteria were centrifuged at 13000 g for 5 min, washed three times with PBS, analyzed on spectrofluorimeter and compared to a standard curve. Like samples were sonicated for 30 min to separate the membrane-bound porphyrin with that incorporated into the cell. Data shown are the average of three trials, each done in duplicate. The upper and lower limit that encompasses 95% of the data is shown on the left of the corresponding column.*

In the binding experiments, the porphyrin bound to the surface of the bacteria could not be distinguished from that incorporated into the bacteria to a level of statistical significance. The concentration/10⁸ cfu after sonication was taken as the most complete representation of porphyrin associated with the bacteria, and it was compared to the inactivation of bacteria by these porphyrins. From the *E. coli* and *Salmonella* binding experiments, the average data for each
of the porphyrins was compared and only that of the TProPyP, which showed no measurable binding to either bacterium, was found to be significantly different from the other porphyrins. Data was analyzed with a paired 2 sided t-test using 0.05 as the lower limit for rejection of the null hypothesis. Actual values for the paired T test can be seen in Appendix 4 (Table A4.1).

Figures 4.2 and 4.3 both show the porphyrin binding to MS2 and PRD-1. The actual values used to make these graphs can be seen in Appendix 4 (Table A4.2). In Figure 4.2 the amount of porphyrin in the supernatant is shown, while in Figure 4.3, the amount present in the viral pellet is shown to compare porphyrin binding to its inactivation of bacteriophages (Tables 4.5 and 4.6).

From Figure 4.2, based on the supernatant, the fixed cationic porphyrin concentrations were lowered more by centrifugation than were their analogs C3PyP and C4PyP. However, from Figure 4.3, where the resuspended pellet is measured, there is conflicting data, which suggest that the C3PyP and C4PyP are more bound to the phage than their analogs. When comparing the C3PyP and C4PyP from Figures 4.2 and 4.3, nearly all of the porphyrin added to the system is accounted for; however, a less complete recovery of the the TBuPyP and TProPyP was acheived. This porphyrin that is unaccounted for could be due to aggregation, which would lower the emitted fluorescence. From the stability experiments in Chapter 3 there was also evidence of porpyrin aggregation (See Appendix 4; Figures A4.1and A4.2)
**Figure 4.2** The porphyrin binding to bacteriophages MS2 and PRD-1 measured via analysis of the supernatant after ultracentrifugation*

*Porphyrins (1 µM) and phage (~10^10 pfu/mL) were centrifuged at 35000 g for 3 hrs, supernatant was analyzed for fluorescence and compared to samples without porphyrin.

**Figure 4.3** The porphyrin binding to bacteriophages measured via the fluorescence of the resuspended pellet after ultracentrifugation*

*Porphyrins (1 µM) and phage (~10^10 pfu/mL) were centrifuged at 35000 g for 3 hrs, supernatant was analyzed for fluorescence and compared to samples without porphyrin. The above is an average of three trials, each done in duplicate. On average, 99.2 % (stdev 0.4) of PRD-1 and 96.3 % (4.33) of MS2 was pelleted.
Table 4.5 MS2 inactivation by fixed and flexible cationic porphyrins*

<table>
<thead>
<tr>
<th>Porphyrin (1 µM)</th>
<th>Log reduction (Standard Deviation)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Light (1 min)</td>
<td>Dark (20 min)</td>
</tr>
<tr>
<td>C3PyP</td>
<td>5.61 (1.44)</td>
<td>0.48 (0.66)</td>
</tr>
<tr>
<td>C4PyP</td>
<td>5.80 (0.55)</td>
<td>0.94 (0.55)</td>
</tr>
<tr>
<td>TProPyP</td>
<td>5.57 (1.07)</td>
<td>1.84 (0.93)</td>
</tr>
<tr>
<td>TBuPyP</td>
<td>5.45 (2.14)</td>
<td>2.35 (0.68)</td>
</tr>
</tbody>
</table>

*Porphyrins and MS2 were irradiated (4.72 mW/cm²) or left in the dark before plating and overnight incubation. Reductions are with respect to the MS2 stock prior to the addition of porphyrin. The above is an average of three trials, each done in duplicate. Standard deviation is shown in parenthesis.

Table 4.6 PRD-1 Inactivation by fixed and flexible cationic porphyrins*

<table>
<thead>
<tr>
<th>Porphyrin (1 µM)</th>
<th>Log reduction of PRD-1 (Standard Deviation)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Light (30 min)</td>
<td>Dark (60 min)</td>
</tr>
<tr>
<td>C3PyP</td>
<td>0.12 (0.19)</td>
<td>0.13 (0.10)</td>
</tr>
<tr>
<td>C4PyP</td>
<td>0.15 (0.01)</td>
<td>0.07 (0.21)</td>
</tr>
<tr>
<td>TProPyP</td>
<td>0.19 (0.07)</td>
<td>0.31 (0.15)</td>
</tr>
<tr>
<td>TBuPyP</td>
<td>0.07 (0.09)</td>
<td>0.13 (0.01)</td>
</tr>
</tbody>
</table>

*Porphyrins and PRD-1 were irradiated (4.72mW/cm²) or left in dark for before plating and overnight incubation. Reductions are with respect to the PRD-1 stock prior to the addition of porphyrin. The above is an average of three trials, each done in duplicate. Standard deviation is shown in parenthesis.

Figures 4.4 and 4.5 show the measurements of singlet oxygen production. A sample chromatogram from the HPLC can be seen in Appendix 5. Figure 4.4 shows the production of the major product of FFA and singlet oxygen, while Figure 4.5 shows the FFA remaining, after 2 hrs of irradiation with the porphyrin. In three of four trials, the TProPyP and the TBuPyP produced more singlet oxygen than their analogs (Figure 4.4). Figure 4.5 supports the above with measurements of the FFA residual. In all four trials, TProPyP and TBuPyP show the least amount of FFA after irradiation when compared to control samples without PS. If more FFA is degraded by a specific porphyrin, then less FFA
should be remaining. So porphyrins with the least FFA after irradiation will have produced larger amounts of singlet oxygen.

**Figure 4.4** Measurement of singlet oxygen- furfuryl alcohol (FFA) product*

*Porphyrin (10 µM) and FFA (10 µM) were irradiated (4.72mW/cm²) for 2 hrs, internal standard was added, and the samples were analyzed via HPLC. The integration of the area under the peak of the FFA-singlet oxygen product was compared to that of the internal standard.
Figure 4.5 Measurement of furfuryl alcohol residual*

*Porphyrin (10 µM) and FFA (10 µM) were irradiated (4.72mW/cm²) for 2 hrs, internal standard was added, and the samples were analyzed via HPLC. The integration of the area under the peak of the FFA with porphyrin was compared to controls with no porphyrin added.

The average data from the singlet oxygen measurements from each porphyrin were also compared with a paired 2 sided t-test using 0.05 as the lower limit for rejection of the null hypothesis. When analyzing the FFA-singlet oxygen product there was no statistically significant difference in that produced by any of the porphyrins; however, the measurement of the FFA residual showed that the C3PyP and C4PyP had a significantly more FFA residual than their fixed cation counterparts, which is indicative of less singlet oxygen production.

Discussion

The purpose of these experiments was to better understand the effect of the cation attachment and flexibility on porphyrin association with and subsequent inactivation of target microorganisms (Specific Aim 4). The C3PyP and C4PyP both showed greater binding to and inactivation of the test bacteria.
than did their analogs TProPyP and TBuPyP. The general trends in porphyrin binding and bacterial inactivation are as follows:

- Binding to *E. coli* $C_4$PyP $\sim$ $C_3$PyP $\sim$ TBuPyP $\gg$ TProPyP
- Inactivation of *E. coli* $C_4$PyP $>$ $C_3$PyP $>$ TBuPyP $\gg$ TProPyP
- Binding to *Salmonella* TBuPyP $\sim$ $C_3$PyP $\sim$ $C_4$PyP $\gg$ TProPyP
- Inactivation of *Salmonella* $C_4$PyP $\geq$ $C_3$PyP $\gg$ TBuPyP = TProPyP

In general the porphyrins with the most binding to the bacteria showed the greatest inactivation. With the *Salmonella*, the $C_3$PyP and $C_4$PyP yielded very close inactivation as well as binding.

Sonication was shown to increase the concentration of the $C_4$PyP and the TBuPyP more than the $C_3$PyP and TProPyP. In the *E. coli* experiment the TBuPyP had nearly the same amount of total bound porphyrin as the $C_3$PyP, but a larger portion of the TBuPyP was incorporated into the cell. The $C_3$PyP still gave a greater inactivation of *E. coli* than the TBuPyP. This shows that increase in porphyrin uptake into the cell does not necessarily increase the potential for inactivation, and it in fact may be negatively correlated with inactivation.

In general the fixed and flexible cationic porphyrins gave a similar yield of singlet oxygen, but exhibited markedly different degrees of binding to the test bacteria. The present data suggest that closer binding to or association with target pathogens is more important than generation of singlet oxygen in determining the efficiency of PDI for bacteria. Furthermore the amount of porphyrin incorporated into the cell seemed to have a negative association with the efficiency of PDI. The latter finding suggests that PDI of bacteria targets the
cellular membranes rather than its genetic material. This finding has been both supported\textsuperscript{11} and opposed\textsuperscript{17} in previous literature.

Viral inactivation was not well correlated to porphyrin binding or singlet oxygen production. All porphyrins rapidly inactivated MS2 (greater than 6 logs reduction in 1 min) and showed little activity against PRD-1 (all less than 0.2 log reduction in 30 min). This low efficiency of PRD-1 inactivation occurred despite the fact that all of the porphyrins, in general, bound more readily to PRD-1 than MS2. For the bacteria, binding and inactivation increased with the flexible cationic porphyrins; however, for the viruses, the fixed and flexible cationic porphyrins gave similar binding and inactivation. There seems to be very different mechanisms for porphyrin inactivation of bacteria and viruses. There were also large disparities in the inactivation of MS2 and PRD-1 by PDI; the greatest difference between the two test viruses is their genetic material, ssRNA and dsDNA, for MS2 and PRD-1 respectively. This major difference suggests that the PDI of viruses could be largely dependent on the genetic material of the target pathogen.
References


Chapter 5: Porphyrin Activation at Wavelengths of Minor Absorbance

Background

To date, investigation and application of photosensitizers (PSs) have largely focused on the development of cancer treatments. The resulting knowledge is often relevant to photodynamic inactivation (PDI). Furthermore, synthetic porphyrins were originally developed for photodynamic therapy (PDT), an approved treatment for several cancers\(^1\)\(^-\)\(^5\), and they have more recently been applied to the disinfection of blood products\(^6\)\(^-\)\(^10\).

In most literature PSs are excited at their maximum absorbance (\(\lambda_{\text{max}}\)) to achieve the most efficient inactivation. Nevertheless, porphyrins have absorbances at longer wavelengths than the Soret that are still at energies capable of generating singlet oxygen. Excitation at these other wavelengths could be preferred in systems where a deeper penetration of light is required, or where a specific type of light is already being used in other processes. Our porphyrins have a maximum absorbance at 411-430 nm, but have four low extinction Q band absorbances between 510 and 650 nm. Activating porphyrins with longer wavelengths of light could be essential for penetrating various media such as turbid solutions and tissues. Longer wavelength light can offer deeper penetration and porphyrin activation in aqueous media that may block out the porphyrin Soret. While drinking water has little turbidity at the disinfection stage,
wastewater can be turbid. The porphyrins in this study also absorb light in the UV range (See Figure 5.1); thus, acceptable levels of singlet oxygen generation at wavelengths with low extinction coefficients would also prove that these porphyrins could be used in conjunction with UV disinfection. The efficacy of porphyrin inactivation of MS2 and the singlet oxygen production were measured using longer wavelengths of light than that of the Soret.

**Materials and Methods**

*Porphyrin Inactivation of MS2*

The inactivation of MS2 incorporated the same experimental setup as described in Chapter 4 with the addition of a yellow light filter that blocked all wavelengths below 450 nm, which encompasses the porphyrin Soret (See Figure 5.1). Porphyrins were irradiated for 5 minutes.

Separately, the inactivation of MS2 (1 mL) by 1 µM C4PyP (the most effective porphyrin in the inactivation of MS2) at 650 nm using a spectrofluorimeter (Perkin-Elmer model 650-10S) was measured. The light intensity of the spectrofluorimeter was ~15 µW/cm², which is less than 1/300 the intensity as that in the test with the light filter described above (4.72 mW/cm²).

*Measurement of Singlet Oxygen*

To measure the singlet oxygen, some amendments were made to the literature-based procedure described in Chapter 4. In PBS buffer 2 mL 10 µM porphyrin and 100 µM FFA was irradiated by the pre-described light and filter for 5 min. An internal standard (IS), phenyl 1-1, 2-ethanediol (100 µM), was added
prior to analysis (Shimadzu HPLC, RP column, flow rate 0.2 mL/min, 70% DIW, 30% methanol).

**Figure 5.1** C4PyP absorbance (red) compared to filter light absorbance (blue).*

*Above is the UV-visible spectrum of 10 µM C4 PyP in 3% SDS (red) shielded by a transparent yellow filter (blue). The full spectrum can be seen on the left, while an expansion of the longer wavelengths can be seen on the right.

**Results**

**Inactivation**

Inactivation of MS2 by all four porphyrins (1µM in PBS) in both the presence and absence of light was examined (Table 5.1). The average log reduction of MS2 in light and dark samples was calculated. In light, all porphyrins yielded a similar inactivation of MS2. The fixed and flexible 4-carbon chain porphyrins were most efficient. TBuPyP showed the highest degree of inactivation in the presence and absence of light with almost a 4-log reduction. Unlike the other porphyrins that had a small (~0.3 log) reduction without light, TBuPyP achieved nearly 90% reduction in each dark trial. TProPyP was the least effective porphyrin under the longer wavelength light.
Table 5.1  MS2 inactivation by porphyrins in PBS with filtered light and in dark*

<table>
<thead>
<tr>
<th>Porphyrin (1µM)</th>
<th>Log inactivation (Standard Deviation)</th>
<th>Log Inactivation Light – Dark</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Light 5 min</td>
<td>Dark ~15 min</td>
</tr>
<tr>
<td>C3PyP</td>
<td>2.90 (0.50)</td>
<td>0.28 (0.33)</td>
</tr>
<tr>
<td>C4PyP</td>
<td>3.16 (0.24)</td>
<td>0.26 (0.02)</td>
</tr>
<tr>
<td>TBuPyP</td>
<td>3.90 (0.54)</td>
<td>0.93 (0.13)</td>
</tr>
<tr>
<td>TProPyP</td>
<td>2.82 (0.49)</td>
<td>0.32 (0.37)</td>
</tr>
</tbody>
</table>

*Porphyrins and MS2 were irradiated (4.72 mW/cm²) while shielded with a light filter. Log reductions are relative to MS2 titers in the absence of porphyrin. Above data is the average of three trials, each with duplicate samples.

Since inactivation of MS2 was observed in the dark, the difference between light and dark inactivation was calculated (Table 5.1, last column). This difference allowed for a separation of the inactivation due to PDI from the inactivation due to the dark toxicity of the porphyrins. By accounting for the dark toxicity of the porphyrins, the variation in inactivation between the porphyrins was reduced. Despite differences in cation position, each of the porphyrins gave similar inactivation of MS2.

The duration of long-wavelength light exposure to reach a 3-log reduction was compared to that of the same porphyrins against MS2 without a light filter (Table 5.2). The time needed for a 3-log reduction was calculated with the assumption that there is a linear relationship between the inactivation of MS2 and the time of light exposure. Evidence of linear inactivation can be seen in Appendix 6. It is evident that longer irradiation times are needed for the porphyrins to inactivate equal amounts of MS2 without irradiation of the Soret. It took roughly 8 to 13 times as long for porphyrins to reach a 3-log reduction with the light filter when compared to the unobstructed light. The ratio between time
with and without the light filter is comparable to the difference in porphyrin activation (based on absorbance).

Table 5.2 Time needed to reach 3-log MS2 reductions with and without light filter*

<table>
<thead>
<tr>
<th>Porphyrin (1µM)</th>
<th>Estimation of 3 Log reduction time (min)</th>
<th>Ratio (with filter: without filter)</th>
<th>Absorbance Ratio (Q band: Soret)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>without light filter</td>
<td>with light filter</td>
<td></td>
</tr>
<tr>
<td>C3PyP</td>
<td>0.4</td>
<td>5.2</td>
<td>12.4</td>
</tr>
<tr>
<td>C4PyP</td>
<td>0.5</td>
<td>4.7</td>
<td>10.4</td>
</tr>
<tr>
<td>TBuPyP</td>
<td>0.5</td>
<td>3.9</td>
<td>7.8</td>
</tr>
<tr>
<td>TProPyP</td>
<td>0.4</td>
<td>5.3</td>
<td>13.3</td>
</tr>
</tbody>
</table>

*Linear estimations of the duration of a 3 log inactivation of MS2 by porphyrins with and without the light filter using data from tables 4.3 and 5.1. The absorbance ratio is the peak height of the largest absorbance not blocked by the filter (minor) compared to that of the Soret.

Separately, the inactivation of MS2 by C4PyP was measured at one specific wavelength of excitation. Since the C4PyP has a minor absorbance at a longer wavelength than the other porphyrins, and it had the most efficient inactivation of MS2, the inactivation of MS2 via the longest wavelength was measured (645 nm; see Figure 5.1) to support the above data. While the singlet oxygen produced under these conditions was below the method detection limit (MDL), which was 2% the area under the curve of the internal standard, it was observed that, under the 645 nm light, the C4PyP was capable of inactivating MS2 to a level of statistical significance (see Table 5.3).
Table 5.3 C4PyP inactivation of MS2 at 645 nm*

<table>
<thead>
<tr>
<th>Test Solution</th>
<th>Percent survival % (standard deviation)</th>
<th>95% upper-lower limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dark</td>
<td>41.4 (22.7)</td>
<td>63.6 -19.1</td>
</tr>
<tr>
<td>Light</td>
<td>6.2 (5.0)</td>
<td>11.1 -1.3</td>
</tr>
</tbody>
</table>

*MS2 and 1µM C4PyP was exposed to 645 nm light for 30 min and compared to the original MS2 stock. The dark exposure consisted of 1µM C4PyP and MS2 placed in the spectrofluorimeter for 30 min without light.

Measurement of Singlet Oxygen

There was a malfunction in the data acquisition of the HPLC, likely due to column fouling, that consistently gave duplicate peaks for pure compounds. While these additional peaks prohibited the quantification of relative amounts of singlet oxygen produced by each porphyrin under filtered light, the proper controls offered proof that each of the porphyrins produced a measurable amount of singlet oxygen (See Figures 5.2-5.9).

Figure 5.2 Control chromatogram of TProPyP (10µM), FFA (100µM) and IS (100µM) without light
**Figure 5.3** Chromatogram of TProPyP (10µM), FFA (100µM) and IS (100µM) with 5 minutes exposure to filtered light

**Figure 5.4** Chromatogram of overlay of figures 5.2 and 5.3*

*Notice the new product peaks around 15 to 20 minutes in the sample with light (blue) as well as an overall reduction in the FFA.
**Figure 5.5** Control chromatogram of FFA and IS with 5 minutes exposure to projector light.

**Figure 5.6** Control chromatogram FFA and IS without light.

**Figure 5.7** Control Chromatogram of TProPyP and IS with 5 minutes exposure to projector light.
Figure 5.8 Chromatogram of overlay of graphs 5.5, 5.6, and 5.7*

The identical control graphs 5.5 and 5.6 show that the IS and FFA do not react under light and that the presence of porphyrins is needed to produce the singlet oxygen – FFA product. Graph 5.7 shows that the porphyrin does not react with the IS under light.

The appropriate controls have shown that the product of singlet oxygen and FFA was formed only by the presence of porphyrin, FFA, and light. The FFA did not react with the IS or light to produce any peaks in the product region (13-23 min). Furthermore, the IS did not react with the porphyrin or singlet oxygen to produce any peaks in the product region.

Discussion

The porphyrins' efficiency with filtered light was compared to the free chlorine disinfection of MS2 (Table 5.4). At similar temperatures and turbidity, the time for a 4-log reduction in the presence of porphyrin was estimated based on an assumption of linear inactivation over time. The CT constant, which is the product of the concentration (mg/L) of the disinfectant and the time needed (min) for specific levels of inactivation, was compared for equal amounts of inactivation. For an equal level of inactivation, the cationic porphyrins have a
much lower CT constant than that of free chlorine, which means that these porphyrins are much more effective against MS2 with the filtered light and less efficient PDI.

**Table 5.4** Comparison of our study to previous literature on chlorine disinfection of MS2

<table>
<thead>
<tr>
<th>System Characteristics</th>
<th>Free Chlorine (Chaidez et. al.)</th>
<th>C3PyP</th>
<th>TProPyP</th>
<th>C4PyP</th>
<th>TBuPyP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Microbe</strong></td>
<td>MS2</td>
<td>MS2</td>
<td>MS2</td>
<td>MS2</td>
<td>MS2</td>
</tr>
<tr>
<td><strong>Disinfectant Concentration</strong></td>
<td>100 mg/L</td>
<td>1.11 mg/L</td>
<td>1.11 mg/L</td>
<td>1.17 mg/L</td>
<td>1.17 mg/L</td>
</tr>
<tr>
<td><strong>Nephelometric Turbidity Units (NTU)</strong></td>
<td>2</td>
<td>0.23</td>
<td>0.23</td>
<td>0.23</td>
<td>0.23</td>
</tr>
<tr>
<td><strong>~ Log Reduction</strong></td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td><strong>Contact Time (min.)</strong></td>
<td>2</td>
<td>6.9</td>
<td>7.1</td>
<td>6.3</td>
<td>5.1</td>
</tr>
<tr>
<td><strong>CT Constant [(mg. min/L)]</strong></td>
<td>200</td>
<td>7.7</td>
<td>7.9</td>
<td>7.4</td>
<td>6.0</td>
</tr>
</tbody>
</table>

The data from these experiments supports that from the previous chapters in that the porphyrins with the more rigid cation attachment proved to be more toxic without irradiation, and all the porphyrins gave a similar inactivation of MS2. The porphyrin inactivation with the light filter proved feasible the application of these porphyrins and other PS with light outside of their maximum absorbance; this was supported by the observed production of singlet oxygen. Even at the highest wavelength, with a lower light intensity that yielded a singlet oxygen production below the method detection limit, significant inactivation of MS2 was observed. In general, if a PS has an absorbance with a low extinction coefficient
of a desired wavelength, then it should be considered for PDI or PDT even if the absorbance is not the $\lambda_{\text{max}}$. 
References


Chapter 6: Conclusion

Review of Specific Aims

The goal of this research was to develop a series of flexible cationic porphyrins as disinfectants for aqueous solutions. The preliminary studies have shown that the flexible cationic porphyrins are potentially more effective than disinfection by free chlorine. In order for this group of porphyrins to become useful in a public health setting, a series of these porphyrins was characterized for use in water treatment. Specific questions were answered so as to better understand the porphyrins’ stability in storage and in use, native state toxicity, and other factors that influence PDI efficiency.

Analogs to the above-described porphyrins were synthesized with the cation positioned at the more-rigid periphery of the porphyrin macrocycle to observe the effects of cation position on porphyrin stability and toxicity, and porphyrin binding to and inactivation of model pathogens relevant to PDI.

The present study offers a proof of concept of these porphyrins’ applicability to the disinfection of water and other aqueous media. The following questions were the focus of this research:
1) What is the optimum chain length for porphyrin inactivation of model pathogens?

   The cationic porphyrins used in previous tests have carried charges at fixed distances from the tetrapyrrole macrocycle. Porphyrins with a flexible alkyl-cation linkage were synthesized to adapt to the various negative charge distributions on the surface of pathogens. The inactivation of *E. coli* by these porphyrins was measured. The most efficient porphyrins were found to be those with a propyl and butyl linkage (C3PyP and C4PyP). This led to the synthesis of rigid cation analogs (TProPyP and TBuPyP) in order to specifically observe the effect of the more flexible cation position on porphyrin stability, toxicity, binding and inactivation.

2) What is the stability of the porphyrins under various conditions that simulate likely real-life use?

   There is now a better understanding of how long the porphyrin maintains its integrity when irradiated as well as in the absence of light. The photostability of four porphyrins with different cation attachment was examined, and the degradation of these porphyrins was confirmed using $^1$H NMR and UV/Vis and mass spectrometry.

   The C3PyP and C4PyP porphyrins degraded much faster than their analogs. This is more than likely due to the pyridinium at the meso position of the analogs which can sterically block nucleophilic attack at the most vulnerable position. The pyridine ring at the meso position of the porphyrins also allows for increased aromaticity, which could contribute to its stability. Even still the C3PyP
and C4PyP porphyrins took longer than 4 hours to completely degrade. This is more than several times the period required for reasonable inactivation of microorganisms.

By monitoring the $^1$H NMR over various lengths of irradiation, it was observed that the pyridine is first to fragment, followed by the break in the porphyrin macrocycle. Although the C3PyP and C4PyP products were not fully defined by the $^1$H NMR and MS, the present observations were in concordance with previous literature that suggests the photoproducts consist of oxidized pyrrole and dipyrrole derivatives, likely accompanied by the aliphatic chain and a pyridinium salt.

Reducing the oxygen in the system reduced porphyrin degradation. Comparison of the partially and fully degraded samples indicated that the initially formed photoproducts were stable over time. The fact that the porphyrin degradation is inhibited by anoxic conditions supports the theory that oxygen is involved in the photodegradation.

3) What is the potential toxicity of the porphyrins and their degradation products?

The toxicity of the parent porphyrins and the photoproducts to model mammalian cells was measured. With the four porphyrins examined, some general trends were observed. The C3PyP and C4PyP were less toxic than their fixed-cation analogs, but had more toxic photoproducts. The TProPyP and TBuPyP, both of which showed little degradation over 24 hours, showed little difference in toxicity between the parent compounds and the irradiated sample.
Although the C3PyP and C4PyP and their photoproducts were nontoxic at the levels suggested for effective disinfection (1µM), the fact that the photoproducts are more toxic is of some concern. The present data suggests that the photoproducts of these porphyrins could be tens of times more toxic than the parent compounds and should be taken into account in risk assessment.

4) How does the attachment of the cation to the porphyrin periphery affect the binding to and subsequent inactivation of specific bacteria and viruses?

The PDI of model pathogens by these porphyrins and their fixed cation analogs was measured to better understand the effect of the mobile cation position. The amount of porphyrin that is bound to the target microbe and the singlet oxygen production was also examined. To meet this aim, a method was developed to observe the viral binding of porphyrins, which has not been previously documented. The porphyrins with the more flexible cation linkage offered greater attachment to bacteria; the porphyrins with the most binding to the bacteria showed the greatest inactivation. The bacterial binding had more influence on inactivation than that of the virus.

It has been shown that closer binding to or association with target pathogens is more important than generation of singlet oxygen in determining the efficiency of PDI of bacteria. Of the porphyrin that was closely associated with or bound to bacteria, that located at the outer membrane was better correlated with inactivation than the porphyrin that was incorporated into the cell. Furthermore, the amount of porphyrin incorporated into the cell seemed to have a negative
association with the efficiency of PDI. The latter finding suggests that PDI of bacteria targets the cellular membranes rather than its genetic material. This finding has been both supported \(^{11}\) and opposed \(^{26}\) in previous literature.

Overall, viral inactivation was not well correlated to porphyrin binding or singlet oxygen production. All porphyrins gave a rapid inactivation of MS2, but showed little activity against PRD-1. This large reduction in the inactivation of PRD-1 occurred despite the fact that all of the porphyrins had greater binding to PRD-1 than MS2. This shows that there are different mechanisms for porphyrin inactivation of bacteria and viruses. There were large disparities in the inactivation of MS2 and PRD-1 by PDI; the greatest difference between these two test viruses is their genetic material, ssRNA and dsDNA, for MS2 and PRD-1 respectively. While this major difference suggests that PDI of viruses could be largely dependent on the genetic material of the target pathogen, both RNA and DNA viruses have been shown to be susceptible to PDI (See Appendix 1).

5) **Will long-wavelength light excite the porphyrins to a level effective in disinfection?**

Porphyrins have a maximum absorbance at 411-430 nm, but have four low extinction Q-band absorbances between 510 and 650 nm. If longer wavelength light can be used to generate singlet oxygen and a biocidal effect, these porphyrins could be used for a wider variety of settings that require deeper light penetration of media. Furthermore, PDI at these longer wavelengths also suggests that these porphyrins could also be used to improve disinfection by UV light. The porphyrins used in this study actually have a greater absorbance of
UV light than the longer wavelengths between 510 and 650 nm; thus, we can assume that they would undergo greater excitation and have a more efficient PDI under UV light than with the longer wavelengths. Even with the filtered light and less excitation, the cationic porphyrins in this study have proven to be more effective than chlorine (from previous literature) in the inactivation of MS2.

The inactivation of MS2 by the porphyrins and filtered light proved feasible the application of these porphyrins and other PSs with light outside of their maximum absorbance; this was supported by the observed production of singlet oxygen. Even at the longest wavelength, with a light intensity that produced singlet oxygen below the threshold for measurement, we observed significant inactivation of MS2. In general, even if a PS has weakly absorbing bands of a desired wavelength, it should be considered for PDI or PDT even if the wavelength is not the $\lambda_{\text{max}}$.

**Future Implications**

The effect of the flexible cation linkage is applicable to all synthetic PSs that are applied to the disinfection of water. Since the pathogens most resistant to modern drinking water treatment have a net negative charge, this flexible linkage could potentially improve the PDI of various types of PSs.

From the vast difference in porphyrin PDI of MS2 and PRD-1, it is evident that all viruses are not equally susceptible to PDI, and target viral pathogens should be independently examined with the PS of choice to predict the efficiency of PDI.
As an added measure of disinfection, the feasibility of PDI should be examined in various stages of drinking water treatment. Since all water treatment facilities are not the same, it is likely that PDI will be more applicable and economical in specific settings. The porphyrins’ range of absorbance and efficiency in turbid media could allow for PDI at various stages of the disinfection process. These PSs would be especially economical in those stages that involve exposure to sun or UV light, or in those where natural irradiation could be achieved.

Due to the increased toxicity of the porphyrin photoproducts, an investigation of strategies for the removal of porphyrins and other PSs is important; however, it is likely that these compounds could be used effectively at levels that would not warrant adverse health effects (no toxicity was observed at 1µM porphyrin which was adequate for the PDI of *E. coli, Salmonella*, and MS2).

The present work is a proof of concept for the use of porphyrins with a flexible cationic linkage to improve PDI. The porphyrins’ absolute lifetime and PDI in environmental samples are areas that need to be further developed.
Appendix 1. A list of previously documented PDI of frank and model pathogens by various PSs

The following list of frank and model pathogens that have been used in studies with photosensitizers and light. All have been used in inactivation studies. Some PSs below have been used in binding studies with bacteria. Inactivation studies varied by the photosensitizer used, the light source, and the duration of exposure. A list of references for the chart follows.

Table A1.1 A list of previously documented PDI of frank and model pathogens

<table>
<thead>
<tr>
<th>Name, strain</th>
<th>Photosensitizer</th>
<th>Inactivation (log inactivation, duration, PS concentration, light type, intensity, wavelength, etc.)</th>
<th>Gram reaction; Enveloped or non-enveloped</th>
<th>genetics</th>
<th>Size, shape, other characteristics</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus aureus</em> MRSA 110</td>
<td>Fixed cationic Porphyrins, Various dyes</td>
<td>7 logs, 20 min, 1µM, 100 mW/cm² 400-800 nm 6 logs, 5-20 J/cm², 1-10 µM 50-400 mW/cm²</td>
<td>Gram +</td>
<td>Circular DNA nucleoid (CDNA)</td>
<td>Spherical/clustered, 0.5- &gt;1µm</td>
<td>12</td>
</tr>
<tr>
<td><em>Enterococcus seriolicida</em></td>
<td>Porphyrins w/ varying # of cations , Zinc pyridinium phthalocyanines</td>
<td>6 logs, 5 min, 8 µM, 6 mW/cm² 3.5 logs, 15 min, 10 µg/mL, 1 mW/cm², 600-700 nm</td>
<td>Gram +</td>
<td>(CDNA)</td>
<td>Marine bacteria</td>
<td>34</td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em> ATCC29212</td>
<td>Meso-phenyl porphyrins w/ varying # of cations iodide-</td>
<td>7 logs, 15 min, 1µM 4 mW/cm², white, (64.8 J/cm²)</td>
<td>Gram +</td>
<td>(CDNA)</td>
<td>Commensal bacteria spherical</td>
<td>5</td>
</tr>
<tr>
<td><em>Listeria monocytogenes</em> LM353</td>
<td>TMPyP</td>
<td>6 logs, 10 min, 5 µM, 7.6 mW/cm², 660 nm</td>
<td>Gram +</td>
<td>(CDNA)</td>
<td>Spherical, includes six species</td>
<td>6</td>
</tr>
<tr>
<td>Fecal Streptococci</td>
<td>TMPyP</td>
<td>4 logs, 5 µM, 60 min, 19 mW/cm²</td>
<td>Gram +</td>
<td>(CDNA)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> ATCC 13706</td>
<td>Meso-phenyl porphyrins w/ varying # of cations iodide-</td>
<td>7 logs, 25 min, 1µM 4 mW/cm², white, (64.8 J/cm²)</td>
<td>Gram -</td>
<td>(CDNA)</td>
<td>Rod shaped</td>
<td>5</td>
</tr>
<tr>
<td><em>E. coli</em> O4</td>
<td>Fixed cationic Porphyrins,</td>
<td>4 logs, 20 min, 1 µM, 100 mW/cm² 400-800 nm 4 logs, 15 min, 1µM, 19 mW/cm²</td>
<td>Gram -</td>
<td>(CDNA)</td>
<td>Rod shaped 2µm by 0.5µm</td>
<td>1, 6, 9, 10</td>
</tr>
<tr>
<td>Organism</td>
<td>Treatment</td>
<td>Conditions</td>
<td>Gram</td>
<td>Notes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>E. coli JM-109</td>
<td>2 logs, 20 min, 4-11 µM, 9 J/cm², 407 nm blue</td>
<td>Gram -</td>
<td>(CDNA)</td>
<td>11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli ATCC 25922</td>
<td>Toluene blue, Rose Bengal bound to poly lysine</td>
<td>6 logs, 0.3 J/cm², 1µM 50-400 mW/cm²</td>
<td>Gram -</td>
<td>Poly lysine bound porphyrin</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>E. coli EC7</td>
<td>Porphyrins w/ varying # of cations</td>
<td>4 logs, 20 min, 1µM, 90 mW/cm² 360-800 nm</td>
<td>Gram -</td>
<td>(CDNA)</td>
<td>12, 13</td>
<td></td>
</tr>
<tr>
<td>E. coli WP2 TM9</td>
<td>TMPyP</td>
<td>5 logs, 30 min, 10 µM 150 mW/cm², white</td>
<td>Gram -</td>
<td>uvrA- and trp-, Ampicillin resistant</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>E. coli TG1</td>
<td>TMPyP</td>
<td>5 logs, 30 min, 10 µM 150mW/cm², white</td>
<td>Gram -</td>
<td>(CDNA)</td>
<td>Transfected with plasmid pUC19</td>
<td>10</td>
</tr>
<tr>
<td>E. coli BS-1</td>
<td>TMPyP</td>
<td>-5 logs, 30 min, 10 µM 150mW/cm², white</td>
<td>Gram -</td>
<td>(CDNA)</td>
<td>hcr-</td>
<td>10</td>
</tr>
<tr>
<td>E. coli DH5</td>
<td>TMPyP</td>
<td>-6 logs, 60 min, 10µg/mL 1mW/cm², 600-700nm</td>
<td>Gram -</td>
<td>(CDNA)</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>E. coli O157:H7</td>
<td>TMPyP</td>
<td>-6 logs, 60 min, 5 µM, 7.6mW/cm, 660 nm</td>
<td>Gram -</td>
<td>(CDNA)</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Pseudomonas aeruginosa strain 12718</td>
<td>Zinc pyridinium phthalocyanines</td>
<td>-4 logs, 60 min, 10µg/mL 1mW/cm², 600-700nm</td>
<td>Gram -</td>
<td>(CDNA)</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Vibrio anguillarum</td>
<td>Porphyrins w/ varying # of cations</td>
<td>-6 logs, 30 min, 8µM, 6 mW/cm²</td>
<td>Gram -</td>
<td>(CDNA)</td>
<td>Marine bacteria</td>
<td>3</td>
</tr>
<tr>
<td>E. coli C3000</td>
<td>Tetra cationic porphyrins w/ flexible cation</td>
<td>-4 logs, 30 min, 1µM, 4.72mW/cm²</td>
<td>Gram -</td>
<td>(CDNA)</td>
<td>Present study</td>
<td></td>
</tr>
<tr>
<td>E. coli F Amp</td>
<td>Tetra cationic porphyrins w/ flexible cation</td>
<td>-4 logs, 30 min, 1µM, 4.72mW/cm²</td>
<td>Gram -</td>
<td>(CDNA)</td>
<td>Present study</td>
<td></td>
</tr>
<tr>
<td>Salmonella LT2</td>
<td>Tetra cationic porphyrins w/ flexible cation</td>
<td>-5 logs, 30 min, 1µM, 4.72mW/cm²</td>
<td>Gram -</td>
<td>(CDNA)</td>
<td>Present study</td>
<td></td>
</tr>
<tr>
<td>Fecal Coliforms</td>
<td>Tri-methyl phenyl substituted porphyrins, TMPyP</td>
<td>-3 logs, 5 µM, 270 min, 9 mW/cm², 600-700nm</td>
<td>Gram -</td>
<td>(CDNA)</td>
<td>7, 14</td>
<td></td>
</tr>
</tbody>
</table>

**Viruses**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Treatment</th>
<th>Conditions</th>
<th>Enveloped</th>
<th>ssRNA</th>
<th>Helical rod shaped</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vesticular Stomatitus virus</td>
<td>Various dyes, Methyl Blue Benzo-porphyrin derivatives</td>
<td>-4-5 logs, 25µM, 240kJ/m² 100mW/cm² red -3 logs, 2 µM, 0.69 J/cm², 3.17mW/cm² LED -2 logs, 4 µg/mL, 20 J/cm², red 600-700nm</td>
<td>Enveloped</td>
<td>ssRNA (-)</td>
<td>Helical rod shaped</td>
</tr>
<tr>
<td>Virus/Pathogen</td>
<td>Type</td>
<td>Natural and synthetic metalated porphyrins</td>
<td>Description</td>
<td>Envelope Status</td>
<td>Viral Genotype</td>
</tr>
<tr>
<td>---------------</td>
<td>------</td>
<td>---------------------------------------------</td>
<td>-------------</td>
<td>----------------</td>
<td>---------------</td>
</tr>
<tr>
<td>HIV Type 1 IIIB</td>
<td>Natural and synthetic metalatedporphyrins (tetraphenyl sulphonate)</td>
<td>&lt;2 log 50 µg/mL, dark</td>
<td>60%</td>
<td>dsRNA</td>
<td>Env proteins</td>
</tr>
<tr>
<td></td>
<td>Methyl Blue derivative</td>
<td>&gt;-3logs, 2 µM, 0.69 J/cm², 3.17mW/cm² LED</td>
<td>-70%, 10 µg/mL, 5 J/cm²</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Di hematoporphyrin Ether</td>
<td>-6logs, 10min, 15 µg/mL, 7mW/cm² fluorescent</td>
<td>-6logs, 20J/cm², 4 µM, 0.001mW/cm²</td>
<td>enveloped</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>dsDNA</td>
</tr>
<tr>
<td>Herpes HSV-1 ATCC 539-VR</td>
<td>Merocyanine 540 Benzo-porphyrin derivatives</td>
<td>-6logs, 10min, 15 µg/mL, 7mW/cm² fluorescent</td>
<td>-6logs, 20J/cm², 4 µM, 0.001mW/cm²</td>
<td>enveloped</td>
<td>dsDNA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatitis A HAV</td>
<td>Fixed porphyrin TMPyP</td>
<td>-6logs, 10 µM, 10min, 2.2mW/cm² 365nm</td>
<td></td>
<td>nonenveloped</td>
<td>ssRNA</td>
</tr>
<tr>
<td>T4-like phage</td>
<td>Meso-phenyl porphyrins w/ varying # of cations iodide-</td>
<td>-7logs, 5µM, 180min 4mW/cm² white</td>
<td></td>
<td>nonenveloped</td>
<td>Linear dsDNA</td>
</tr>
<tr>
<td>T7 Phage</td>
<td>Glycosylated tetra phenyl porphyrin</td>
<td>-6logs, 2 µM, 80mW/cm², 0.2KJ/cm², 400-650nm</td>
<td></td>
<td>nonenveloped</td>
<td>DNA</td>
</tr>
<tr>
<td>Lambda phage</td>
<td>TMPyP</td>
<td>0.7 logs, 660 mn</td>
<td></td>
<td>nonenveloped</td>
<td>Linear dsDNA</td>
</tr>
<tr>
<td>Poliovirus</td>
<td>Methylene Blue</td>
<td>-2.5 logs, 5 min, 2mW/cm², 670nm, 13 µM, pH10</td>
<td></td>
<td>nonenveloped</td>
<td>ssRNA (+)</td>
</tr>
<tr>
<td>Feline Leukemia Virus</td>
<td>Benzo-porphyrin derivatives</td>
<td>-1log, 0.1 µg/mL, 20 J/cm², red 600-700nm</td>
<td></td>
<td>enveloped</td>
<td>ssRNA</td>
</tr>
<tr>
<td>MS2</td>
<td>TMPyP, Flexible cationic porphyrins</td>
<td>-4logs, 10 µM,1min, 2.2mW/cm² 365nm</td>
<td></td>
<td>nonenveloped</td>
<td>ssRNA</td>
</tr>
<tr>
<td>PRD-1</td>
<td>TMPyP, Flexible cationic porphyrins</td>
<td>&lt;1log, 30min, 1µM, 4.72mW/cm²</td>
<td></td>
<td>nonenveloped</td>
<td>ds DNA</td>
</tr>
</tbody>
</table>

**Fungi**

<table>
<thead>
<tr>
<th>Fungus</th>
<th>Type</th>
<th>Description</th>
<th>Envelope Status</th>
<th>Viral Genotype</th>
<th>Antiviral Mechanism</th>
<th>Data Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saprolegnia spp</td>
<td>Fixed cationic Porphyrins,</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Candida albicans ATCC 18804</td>
<td>Toluene blue, Rose Bengal bound to poly lysine</td>
<td></td>
<td></td>
<td>Diploid</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Yeast Saccharomyces cerevisiae</td>
<td>Meso-arylglycosyl porphyrins (TMPyP +sugar)</td>
<td>3 logs, 10µM, 30 min, 50mW/cm²</td>
<td></td>
<td></td>
<td></td>
<td>26</td>
</tr>
<tr>
<td>Parasites</td>
<td>Tetra cationic phthalocyanine</td>
<td>1 log, 5 min, 1 µM 50mW/cm², white</td>
<td>Protozoa</td>
<td>15-35µm, oval or triangular when moving</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------------------</td>
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<td>------------------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acanthamoeba palestinensis</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ascaris lumbricoides</td>
<td>Fixed porphyrin TMPyP tosyl</td>
<td>48%, 30 min, 10 µM, 0.005mW/cm² halogen</td>
<td>Hematode</td>
<td>Giant roundworm, eggs are 45-75µm long 35-50µm thick</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Taenia eggs</td>
<td>Fixed porphyrin TMPyP</td>
<td>20%, 30 min/10 µM, 0.005mW/cm² halogen</td>
<td></td>
<td>Tapeworm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T. cruzi</td>
<td>Phthalocyanines</td>
<td>&gt;4logs, 10 min, 2µM</td>
<td>Euglenoid trypanosomes</td>
<td>Trypomastigote</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P falciparum</td>
<td>Phthalocyanines</td>
<td>92% inhibition, 10 min, 2µM</td>
<td>Protozoa</td>
<td>Ring-stage Brazilian isolate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vahlkampfia hartmannii cysts</td>
<td>Al(III)Phthalocyanine</td>
<td>5 log, 10 min, 3 µM, 100mW/cm²</td>
<td>Protozoa</td>
<td>Fresh Water Amoeba,</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Appendix 2. Additional $^1$H NMR and MS spectra of porphyrins before and after exposure to light

In chapter 3 we synthesize the TBuPyP and the TProPyP. Structures were confirmed with $^1$H NMR (shown below). These porphyrins showed no change in their $^1$H NMR spectra after 24 hours irradiation.

**Figure A2.1** $^1$H NMR of TProPyP before (a) and after (b) 24 hours of exposure to light.

(a)

(b)
Figure A2.2 $^1$H NMR of TBuPyP before (a) and after (b) 24 hours of exposure to light

(a)

(b)
Figures A2.3 through A2.6 show the mass spectra of the porphyrins before and after 24 hours exposure to light.

**Figure A2.3** The mass spectra of C3PyP (a) and its photoproduct (b)
Figure A2.4 The mass spectra of C4PyP (a) and its photoproduct (b)

(a)

C4PyPnew #4 14.09 #20-356 RT: 0.57-10.00 AV: 337 NL: 1.20E6
T: + p ESI Full ms [150.00-2000.00]

(b)

C4PyPalt #20-175 RT: 0.57-4.96 AV: 156 NL: 2.94E6
T: + p ESI Full ms [150.00-2000.00]
Figure A2.5 The mass spectra of the TProPyP before (a) and after (b) 24 hours of exposure to light

(a)

TProPyPnew 4_14_09 #20_286 RT: 0.56-8.01 AV: 267 NL: 2.42E6
T: + p ESIFull ms [150.00-2000.00]

(b)

TProPtrial2 #26-504 RT: 0.56-10.91 AV: 479 NL: 9.91E6
T: + c ESIFull ms [150.00-1500.00]
Figure A2.6 The mass spectra of the TBuPyP before (a) and after (b) 24 hours of exposure to light.
Appendix 3. Sample calculations for loss of water during the stability experiment in Chapter 3

Sample calculations for one experiment are shown with a starting volume of 4.88mL of C4PyP and 4.45mL of TBuPyP after the t=0 sample. The volume lost to evaporation at any time is the difference between the mass after a sample for the previous sample time and the mass before the sample of the current sampling time. This mass in mg translates to the volume in mL loss to evaporation. The after sample volume at a given time is the after sample volume from the previous sample time minus .210mL – (loss of evaporation). (See Table A3.1)

**Table A3.1** The data collected in order to account for water evaporation in the formation of porphyrin products

<table>
<thead>
<tr>
<th>Time</th>
<th>Before Sample Mass mg</th>
<th>After Sample mass mg</th>
<th>After sample volume mL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C4PyP</td>
<td>TBuPyP</td>
<td>C4PyP</td>
</tr>
<tr>
<td>4</td>
<td>18.6485</td>
<td>18.9275</td>
<td>18.4194</td>
</tr>
<tr>
<td>6</td>
<td>18.3651</td>
<td>18.6369</td>
<td>18.1325</td>
</tr>
<tr>
<td>24</td>
<td>17.8023</td>
<td>18.0143</td>
<td>17.5757</td>
</tr>
</tbody>
</table>

Concentration of test sample \( (t) = \frac{(\text{Conc})_{t-1}(\text{volume}_{after\ sample})_{t-1}}{(\text{volume}_{new})} \)

Where the new volume = \( (\text{volume}_{after\ sample})_{t-1} - (\text{volume}_{evaporation}) \)

At T=1 hour for C4PyP, the loss of water due to evaporation is as follows:
(Mass of $t_0$ After sample)-(Mass of $t_1$ Before Sample) = 19.2427-19.2038 = 0.0389

So, the concentration of the C4PyP test solution after one hour of evaporation is

\[(1\text{mM})(4.88\text{mL})/4.8411\text{mL}) = 1.008\text{mM}\]

This sample would then be diluted with 1008µL to achieve the 10µM solution for the absorbance measurement. Furthermore 1.008 mM will be used to calculate the concentration of the solution at the next sampling time.
Appendix 4. Actual values from porphyrin binding in Chapter 4

From the *E. coli* and *Salmonella* binding experiments, the average data for each of the porphyrins was compared and only that of the TProPyP, which showed no measurable binding to either bacterium, was found to be significantly different from the other porphyrins. Data was analyzed using a paired 2 sided t-test using 0.05 as the lower limit for rejection of the null hypothesis (Table A4.1)

**Table A4.1** The paired t-test values for each porphyrin*

<table>
<thead>
<tr>
<th>Porphyrin</th>
<th>C3PyP</th>
<th>C4PyP</th>
<th>TProPyP</th>
<th>TBuPyP</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3PyP</td>
<td>N/A</td>
<td>0.24</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>C4PyP</td>
<td>0.36</td>
<td>N/A</td>
<td>0.03</td>
<td>0.51</td>
</tr>
<tr>
<td>TProPyP</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>N/A</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>TBuPyP</td>
<td>0.85</td>
<td>0.73</td>
<td>0.02</td>
<td>N/A</td>
</tr>
</tbody>
</table>

*The *E. coli* results are shown in green (top right) and the *Salmonella* results are shown in blue(bottom left)

In Chapter 4, the data from the porphyrin binding to MS2 and PRD-1, measured as the percent of porphyrin fluorescence, was presented as bar graphs. Table A4.2 shows the actual values that were used to produce those graphs.
Table A4.2 The binding to MS2 and PRD-1 measured as percent fluorescence when compared to controls*

<table>
<thead>
<tr>
<th>Sample</th>
<th>C3PyP % (std dev)</th>
<th>C4PyP % (std dev)</th>
<th>TProPyP % (std dev)</th>
<th>TBuPyP % (std dev)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS2 SN</td>
<td>85.8 (5.41)</td>
<td>88.4 (7.96)</td>
<td>65.9 (2.84)</td>
<td>63.3 (4.22)</td>
</tr>
<tr>
<td>MS2 Pellet</td>
<td>16.5 (4.11)</td>
<td>17.2 (2.45)</td>
<td>17.2 (3.42)</td>
<td>8.44 (2.26)</td>
</tr>
<tr>
<td>PRD-1 SN</td>
<td>87.2 (3.64)</td>
<td>89.3 (14.1)</td>
<td>69.3 (4.22)</td>
<td>61.6 (5.04)</td>
</tr>
<tr>
<td>PRD-1 Pellet</td>
<td>20.0 (4.31)</td>
<td>22.3 (5.22)</td>
<td>16.3 (3.16)</td>
<td>15.4 (2.18)</td>
</tr>
<tr>
<td>Control SN</td>
<td>95.5 (1.68)</td>
<td>94.6 (2.16)</td>
<td>95.6 (0.334)</td>
<td>100.6 (1.98)</td>
</tr>
<tr>
<td>Control pellet</td>
<td>2.54 (0.610)</td>
<td>1.29 (1.05)</td>
<td>3.02 (2.00)</td>
<td>2.26 (0.417)</td>
</tr>
</tbody>
</table>

*Fluorescence was measured in the supernatant (SN) and resuspended pellet. Porphyrins (1 µM) and phage (~10^10 pfu/mL) were centrifuged at 35000 g for 3 hours, supernatant was analyzed for fluorescence and compared to control samples with porphyrin and TSB. The above is an average of three trials done in duplicate. On average, 99.16 % (stdev 0.3695) of PRD-1 and 96.3 % (4.33) of MS2 was pelleted.

As a control, to ensure that the bacteriophages were pelleted, viral stocks were plated before ultracentrifugation and after resuspension in equal volume to measure the percentage of virus pelleted by our methods (35000 g for 3 hours). We found that on average, 99.16 % (stdev 0.3695) of PRD-1 and 96.3 % (4.33) of MS2 was pelleted.

In Chapter 4, there was not a complete recovery of the TProPyP or TBuPyP from analysis of the supernatant and resuspended pellet. It was suggested that the porphyrin fluorescence was reduced by aggregation. In previous degradation experiments TMPyP, a fixed cationic porphyrin similar to TProPyP and TBuPyP, showed an initial increase in absorbance when exposed to light. This increase was thought to be from the disaggregation of porphyrin molecules via the generation of singlet oxygen. (See Figure 4A.1) A similar initial increase in the Soret was also observed for the TProPyP and the TBuPyP porphyrins (See Figure A4.2)
**Figure A4.1** The increase in the absorbance of the Soret of TMPyP over time

![Graph showing the increase in absorbance over time for TMPyP.]

\[ y = -0.003x^2 + 0.040x + 1.714 \]
\[ R^2 = 0.962 \]

\[ y = 0.032x + 1.127 \]
\[ R^2 = 0.977 \]

*Measurements were taken at various times of irradiation up to 24 hrs and diluted to 10 µM prior to analysis.

**Figure A4.2** The absorbance of the Soret of the TProPyP and TBuPyP porphyrins over time

![Graph showing the absorbance changes over time for TProPyP and TBuPyP.]

*Measurements were taken at various times of irradiation up to 24 hrs and diluted to 10 µM prior to analysis.
Appendix 5. A sample HPLC chromatogram from measurements of singlet oxygen production in Chapter 4

In Chapter 4 the singlet oxygen production of each porphyrin was measured using an HPLC and UV detector.

Figure A5.1 A sample chromatogram of the porphyrin, photoproduct, furfuryl alcohol (FFA), and the internal standard
Appendix 6. Evidence of linear inactivation

In Chapter 5, an assumption was made of a linear inactivation of the test pathogens over the time period of irradiation. This assumption was based on the following graph, which shows that the inactivation of *E. coli* and *Salmonella* by C3 PyP and C4PyP fit well to the linear models. The model for each porphyrin /combination is shown to the right of its description in the legend.

Figure A6.1 Graphs showing linear inactivation of *E. coli* and *Salmonella* by C3PyP and C4PyP
References


10 G. Valduga, B. Breda, G. M. Giacometti, G. Jori and E. Reddi, Photosensitization of Wild and Mutant Strains of Escherichia coli by meso-Tetra


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