

STUDIES TOWARDS ZINC(II) BISDIPICOLYLAMINE COMPLEX BEARING
MOLECULES, LIPOSOMES AND GLASS MICROSPHERES FOR THE
INACTIVATION AND SEQUESTRATION OF BACTERIAL CELLS

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY



BY
HATİCE TUĞBA SELVİ

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
CHEMISTRY

JULY 2023

Approval of the thesis:

**STUDIES TOWARDS ZINC(II) BISDIPICOLYLAMINE COMPLEX
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FOR THE INACTIVATION AND SEQUESTRATION OF BACTERIAL
CELLS**

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ABSTRACT

STUDIES TOWARDS ZINC(II) BISDIPICOLYLAMINE COMPLEX BEARING MOLECULES, LIPOSOMES AND GLASS MICROSPHERES FOR THE INACTIVATION AND SEQUESTRATION OF BACTERIAL CELLS

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July 2023, 122 pages

The widespread and frequent unnecessary use of antibiotics has resulted in the frequent and continuous emergence of bacterial strains resistant to antibiotics in clinical use. As a result, antibiotics are becoming obsolete, and now pathogenic bacterial strains exist that do not respond to known antibiotics. Antibiotic-resistant bacteria are one of the most important risks to public health.

There are several approaches that could be taken to tackle this problem. One approach is to enhance the selectivity of antibiotic agents by conjugating them with bacterial targeting groups to widen their therapeutic window. Zinc(II) bisdipicolylamine ($\text{Zn}_2(\text{BDPA})$) complexes, which are known to bind to bacterial membranes and walls, can be used for this purpose. Another approach is antibacterial photodynamic therapy (PDT). In PDT a photosensitizer (e.g., porphyrins) is used to absorb light and generate singlet oxygen ($^1\text{O}_2$) which is highly toxic towards bacterial cells, although this toxicity is not cell-specific. The probability of emergence of widespread bacterial resistance towards $^1\text{O}_2$ is known to be low. Yet another

potential approach is to remove bacterial from liquids, particularly blood, and thus reduce the infective potential.

The aims of the studies summarized in this thesis are three-fold: The first objective is to evaluate the PDT efficacy of a number of $Zn_2(BDPA)$ complex bearing porphyrin derivatives against a number bacterial strains. The second objective is to develop $Zn_2(BDPA)$ complex bearing liposomes and to explore their antibiotic potential. The third is to develop $Zn_2(BDPA)$ complex bearing glass microspheres for sequestration of bacterial cells from liquids, blood in particular.

Keywords: Porphyrin, bisdipicolylamine ligand, photodynamic therapy, glass microspheres, liposomes

ÖZ

BAKTERİYEL HÜCRELERİN İNAKTİVASYONU VE AYRIŞTIRILMALARI İÇİN ÇİNKO(II) BİSDİPİKOLİLAMİN KOMPLEKSİ TAŞIYAN MOLEKÜLLER, LİPOZOMLAR VE CAM MİKROKÜRELERE YÖNELİK ÇALIŞMALAR

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Temmuz 2023, 122 sayfa

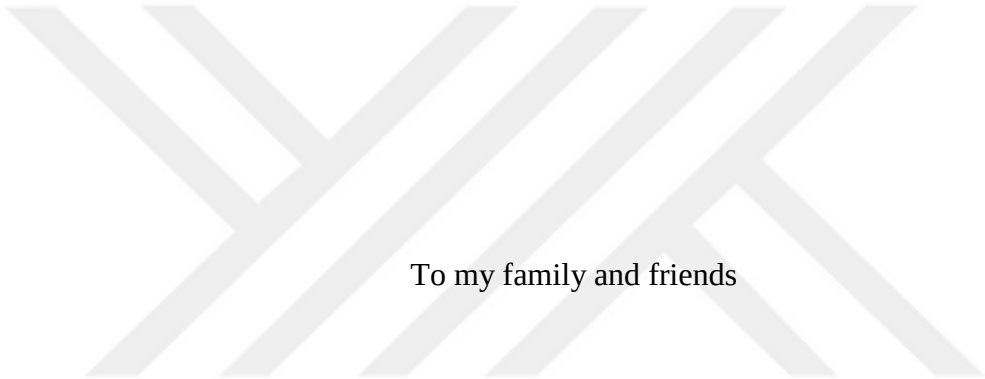
Antibiyotiklerin yaygın ve sıklıkla gereksiz kullanımı, klinik kullanımda antibiyotiklere dirençli bakteri suşlarının sık ve sürekli olarak ortaya çıkmasına neden olmuştur. Sonuç olarak, çoğu antibiyotik sınıfları etkinliklerini yitirmekte ve artık bilinen antibiyotiklere yanıt vermeyen patojenik bakteri türleri ortaya çıkmaktadır. Antibiyotiğe dirençli bakteriler halk sağlığı için en önemli risklerden biridir.

Bu sorunu çözmek için çeşitli yaklaşımlar geliştirilmektedir. Bir yaklaşım, terapötik pencerelerini genişletmek için antibiyotikleri bakteriyel hedefleme grupları ile birleştirerek bu ajanların seçiciliğini arttırmaktır. Bakteriyel hücre zarlarına ve duvarlarına bağlandığı bilinen çinko(II) bisdipikolilamin ($Zn_2(BDPA)$) kompleksleri bu amaçla kullanılabilir. Diğer bir yaklaşım ise antibakteriyel fotodinamik terapidir (PDT). PDTde, ışığı soğurmak ve bakteri hücrelerine karşı oldukça toksik olan singlet oksijen ($^1O_2$) üretmek için bir fotoduyarlaştırıcı (örneğin, porfirinler) kullanılır, ancak bu toksisite bakteriyel hücrelere özgü değildir. $^1O_2$ 'ye karşı yaygın

bakteri direncinin ortaya çıkma olasılığı düşüktür. Yine başka bir potansiyel yaklaşım, bakterileri sıvılardan, özellikle kandan uzaklaştırmak ve böylece enfektif potansiyeli azaltmaktır.

Bu tezde özetlenen çalışmaların amaçları üç yönlüdür: Birinci amaç, bir dizi $Zn_2(BDPA)$ kompleksi taşıyan porfirin türevlerinin bir dizi bakteri suşuna karşı PDT etkinliğini değerlendirmektir. İkinci amaç, $Zn_2(BDPA)$ kompleksi taşıyan lipozomları geliştirmek ve antibiyotik potansiyellerini keşfetmektir. Üçüncüsü, bakteri hücrelerinin sıvılardan, özellikle kandan ayrılması için $Zn_2(BDPA)$ kompleksi taşıyan cam mikroküreler geliştirmektir.

Anahtar Kelimeler: Porfirin, bisdipikolilamin ligandı, fotodinamik terapi, cam mikroküreler, lipozomlar



To my family and friends

ACKNOWLEDGMENTS

First and foremost, I would like to express my deepest gratitude to Assoc. Prof. Dr. Serhan Türkyılmaz for his invaluable guidance, generosity, and unwavering support throughout this journey. His encouragement during challenging times has taught me the importance of persistence, a vital quality for any researcher. I am truly grateful for the privilege of being a part of his research group.

I would also like to extend my sincere thanks to Assoc. Prof. Dr. Salih Özçubukçu, Assoc. Prof. Dr. Özgül Persil Çetinkol, Assoc. Prof. Dr. Urartu Özgür Şafak Şeker, and Assoc. Prof. Dr. Çağdaş Devrim Son for their time and expertise in guiding me in my thesis. Their valuable input and dedication have greatly contributed to the success of my work.

I really appreciate the time and valuable comments Prof. Dr. İrem Erel Göktepe and their research group shared with me during the DLS measurements. I am grateful for their contribution.

I would like to express my heartfelt gratitude to Fulya Şahin, who has been an incredible lab mate to work with and a wonderful friend who has always been by my side. Her unwavering support, encouragement, and friendship have made this research journey more enjoyable and memorable.

I would like to extend my sincere thanks to Doğan Akbulut, and Rozerin Sancar for their unwavering support throughout the process of completing this thesis. I am deeply indebted to all the members of STRG, including Ozan Yılmaz, Şafak Tekir, Sena İpek Baydili, Deniz Öztürker, and many others, for their collaboration and solidarity. Their contributions have played a significant role in the success of this research.

Special appreciation to Kübra Erden, whose presence has brought joy and support to my days. I would also like to express my gratitude to all the members of DRG for the moments of laughter we have shared.

I am grateful for the financial support provided by The Scientific and Technological Research Council of Turkey, TUBITAK with project number 122Z027. I would also like to thank TUBITAK for the scholarship awarded through this project.

To my dear mother and father, and to my brothers, thank you for not only being my siblings but also my friends and for sharing the most memorable moments of our lives together. Without your presence and influence, I wouldn't be standing where I am today.

I am incredibly fortunate to have never felt alone, thanks to Ayşe Nur Yılmaz, whose friendship has spanned eight years filled with laughter and tears. I cannot imagine how my days would be at METU without her, and I am grateful for her presence in my life.

To Alper Şahıstan, I can't wait to see the next chapter of our lives together. I cannot fathom what my life would be like without you.

I would like to express my gratitude to Zümral Çankaya, who has become my second family in Ankara and has always been there to help me in any possible way.

I would like to express my gratitude to my cat, Fındık, for being the most amazing pet anyone could have.

TABLE OF CONTENTS

ABSTRACT	v
ÖZ.....	vii
ACKNOWLEDGMENTS	x
TABLE OF CONTENTS	xii
LIST OF TABLES	xv
LIST OF FIGURES.....	xvii
LIST OF ABBREVIATIONS	xx
LIST OF SYMBOLS.....	xxii
1 INTRODUCTION.....	23
1.1 General Properties of Bacteria.....	24
1.2 Antibiotic Resistance	26
1.3 Differences Between Mammalian and Bacterial Cell Membranes.....	27
1.4 Targeting Strategies	28
1.5 Treatment of Antibiotic-Resistant Bacteria	29
1.6 Photodynamic Therapy	30
1.7 Porphyrins as PDT Agents.....	32
1.8 Heavy Atom Effect	34
1.9 Liposomes.....	35
1.9.1 Lipids.....	35
1.9.2 Micelles and Bilayers	38
1.9.3 Liposomal Encapsulation	40
1.10 Application on Bacteria.....	43

1.10.1	Bacteria Handling	43
1.11	Bacteremia	45
1.12	Objectives of These Studies.....	47
2	EXPERIMENTAL	51
2.1	General Information	51
2.2	Bacterial Inactivation Experiments	52
2.2.1	Preparation of Bacterial Suspensions for all Experiments.....	52
2.2.2	Light Characterization for Photodynamic Inactivation Experiments	53
2.2.3	First Generation Approach for Preparation of Bacterial Suspensions for Irradiation and for Dark Treatment	54
2.2.4	Irradiation of Bacterial Suspensions in the Presence of Oxygen	55
2.2.5	Zinc Toxicity on Bacterial Cells	57
2.2.6	Second Generation of Preparation of Bacterial Suspensions for Irradiation and for Dark Treatment.....	58
2.3	Broth Microdilution Experiments	60
2.3.1	Broth Microdilution Procedure for Antibiotic Essays	60
2.3.2	Broth Microdilution for Photodynamic Inactivation on Bacteria	61
2.3.3	Metalation of PDT Agent apo-2.....	62
2.3.4	Broth Microdilution for Testing of Photodynamic Inactivation of Bacteria	62
2.4	Liposome Preparation	63
2.4.1	Thin Film Formation.....	63
2.4.2	Lipid Film Hydration and Liposome Formation.....	64
2.5	Glass Derivatizations.....	66
2.5.1	Glass Microspheres	66

2.5.2	Strategy I for Glass Derivatization	66
2.5.3	Strategy II for Glass Microsphere Derivatization	73
3	RESULTS AND DISCUSSION.....	79
3.1.1	Antimicrobial Photodynamic Therapy Experiments	79
3.2	Broth Microdilution Experiments for Antibiotic Efficacy Determination	92
3.2.1	Broth Microdilution Experiments for Antibacterial Photodynamic Inactivation Efficacy Determination	95
3.3	Rifampicin Liposomes	101
3.4	Studies Towards Zn ₂ BDPA Complex Bearing Glass Microspheres for the Sequestration of Bacterial Cells	103
3.4.1	Activation of Glass Microspheres	103
3.4.2	Amine-Derivatization of Activated Glass Microspheres Using APTES	103
3.4.3	Calculation of Surface Amine Group Density of GMs after APTES Treatment Using the Kaiser Test	105
3.4.4	Reaction of GM-Amine with Succinic Anhydride	106
3.4.5	Synthesis of BDPA-Amine Ligand (8).....	107
3.4.6	Synthesis of the BDPA-Acid (12) Ligand.....	108
3.4.7	BDPA Derivatized Glass Microspheres	109
4	CONCLUSION	113
	REFERENCES	117
	APPENDICES	141
A.	NMR Spectra	141
B.	Dark Control Experiments for aPDT of 1d.....	144

LIST OF TABLES

TABLES

Table 1. Lipid composition for liposomes containing 2 mol% Zn ₂ BDPA-CF ₃ -Porphyrin (2), 3.32 μmol total lipid.	65
Table 2. Lipid composition for liposomes containing 2 mol% Zn ₂ BDPA-CF ₃ -Porphyrin (2), 3.32 μmol total lipid. No cholesterol was present in this formulation.	65
Table 3. Lipid composition for liposomes containing 5 mol% Zn ₂ BDPA-CF ₃ -Porphyrin (2), 3.32 μmol total lipid. No cholesterol. This formulation gives [Zn ₂ BDPA-CF ₃ -Porphyrin] = 0.166 mM in 1 mL liposomal dispersion.	66
Table 4. Lipid composition for liposomes containing 10 mol% Rifampicin and 5 mol% Zn ₂ BDPA-Chol, 10 μmol total lipid. No cholesterol. This formulation gives [Rifampicin] = 1 mM in 1 mL liposomal dispersion.	66
Table 5. Light and dark toxicity values of molecules investigated in this study against <i>S. aureus</i> ATCC 25923.	92
Table 6. Antibiotic MIC value observed for <i>P. aeruginosa</i> ATCC 9027 through broth microdilution.	94
Table 7. Antibiotic MIC values observed for <i>P. aeruginosa</i> ATCC 27853 through broth microdilution.	94
Table 8. Antibiotic MIC values observed for <i>S. aureus</i> ATCC 25923 through broth microdilution.	94
Table 9. Antibiotic MIC values observed for <i>E. coli</i> ATCC 25922 through broth microdilution.	94
Table 10. Antibiotic MIC values observed for <i>B. subtilis</i> ATCC 6633 through broth microdilution.	94
Table 11. Antibiotic MIC values observed for <i>B. cereus</i> ATCC 14579 through broth microdilution.	95

Table 12. MIC values of **2** against various bacterial species in the presence and
absence of light.....98



LIST OF FIGURES

FIGURES

Figure 1. Gram (+) and Gram (-) cell membranes. ¹	25
Figure 2. Chronology of antibiotics being introduced into clinical use (below the black line) and the chronology of the emergence of antibiotic resistance to those antibiotics (above the black line). Source: Centers for Disease Control and Prevention.	27
Figure 3. Interaction between a Zn ₂ BDPA complex and the negatively charged phospholipid, phosphatidylglycerol.	29
Figure 4. Structures of phosphatidylglycerol, lipid A, and lipoteichoic acid. ³¹	29
Figure 5. Simplified Jablonski Diagram ³¹	31
Figure 6. The general representation of a porphyrin molecule and its <i>meso</i> sites. .	32
Figure 7. Different classes of lipids. ⁷⁷	36
Figure 8. Structures of Micelles and Liposomes.	39
Figure 9. Schematic Scheme of Liposomal Encapsulation of drugs.....	41
Figure 10. Schematic diagram of a 96 well-plate after Broth Microdilution experiment.....	45
Figure 11. Schematic summary of bacteremia and potential consequences.	46
Figure 12. Diagram of preparation of bacterial suspensions for irradiation and dark treatment in first generation approach	55
Figure 13. Diagram of preparation of bacterial suspensions for irradiation and dark treatment in second-generation approach.	59
Figure 14. Schematic diagram of broth microdilution procedure.	61
Figure 15. Schematic diagram of liposome preparation by thin film hydration and extrusion.....	64
Figure 16. Schematic diagram of liposomes.....	65
Figure 17. Photodynamic inactivation and dark toxicity of Iodo-Zn ₂ BDPA-porphyrin(1d) on <i>E. coli</i> . The error bars represent the standard deviation from at least three independent experiments.	81

Figure 18. Photodynamic inactivation and dark toxicity of Iodo-Zn ₂ BDPA-porphyrin (1d) on <i>S. aureus</i> . The error bars represent the standard deviation from at least three independent experiments.....	82
Figure 19. The effect of [Zn(NO ₃) ₂] on <i>E.coli</i> Top10 viability. The error bars represent the standard deviation from at least three independent experiments	83
Figure 20. Concentration-dependent light and dark toxicities of Zn ₂ BDPA-porphyrin derivative 1d against Gram (+) bacterial species <i>S. aureus</i> . The error bars represent the standard deviation from at least three independent experiments. An asterisk indicates a statistically significant difference at $p \leq 0.05$	85
Figure 21. Concentration-dependent light and dark toxicities of Zn ₂ BDPA-porphyrin derivative 1d against Gram (+) bacterial species <i>B. cereus</i> . The error bars represent the standard deviation from at least three independent experiments. An asterisk indicates a statistically significant difference at $p \leq 0.05$	87
Figure 22. Concentration-dependent light and dark toxicities of Zn ₂ BDPA-porphyrin derivative 1d against Gram (-) bacterial species <i>E. coli</i> . The error bars represent the standard deviation from at least three independent experiments. An asterisk indicates a statistically significant difference at $p \leq 0.05$	88
Figure 23. Concentration-dependent light and dark toxicities of Zn ₂ BDPA-porphyrin derivative 1d against Gram (-) <i>P. aeruginosa</i> . The error bars represent the standard deviation from at least three independent experiments. An asterisk indicates a statistically significant difference at $p \leq 0.05$	89
Figure 24. The confocal microscopy analysis of GFP expressing <i>E. coli</i> cells treated with 1a (Zeiss LSM 800 at 63x magnification).....	91
Figure 25. Fragments for which light and/or dark toxicity was investigated using the broth microdilution method.	92
Figure 26. Porphyrin HOMOs and LUMOs. (B) shows the Q band and Soret(B) band transitions. ¹⁴¹	96
Figure 27. UV-Vis spectrum of apo-2 . (insert Q bands).....	97
Figure 28. UV-Vis spectrum of 2 . (insert Q bands)	98

Figure 29. DLS size distribution graph for the first liposomal formulation (Lipo-1) for the encapsulation of 2	99
Figure 30. DLS size distribution graph for the second liposomal formulation (Lipo-2) for the encapsulation of 2	100
Figure 31. DLS size distribution graph for the third liposomal formulation (Lipo-3) for the encapsulation of 2	101
Figure 32. DLS size distribution graph for a liposomal formulation of rifampicin.	102
Figure 33. Picture of GM-1(left) and GM-Amine (right) after ninhydrin test.....	104
Figure 34. Calibration curve for the Kaiser test (Slope: 3.8975; Intercept: 0.056; R ² : 0.999).	105
Figure 35. ATR-FTIR spectra of GM-Amine and GM-Acid.	107
Figure 36. ATR-FTIR spectra of GM-Amine and BDPA-GM-II.....	110
Figure 37. Bright field micrographs (10x) of GM A , GM-Amine C , BDPA-GM-II E , and fluorescence micrographs (10x, GFP filter) of GM B , GM-Amine D , BDPA-GM-II F after treatment with GFP-expressing <i>E. coli</i> K12 and washing.	111

LIST OF ABBREVIATIONS

ABBREVIATIONS

aPDT	antimicrobial photodynamic therapy
PDT	photodynamic therapy
PS	photosensitizer
ROS	reactive oxygen species
CMC	critical micelle concentration
NP	nanoparticle
SUV	small unilamellar vesicles
LUV	large unilamellar vesicles
MLV	multilamellar vesicles
MVV	multivesicular vesicles
MIC	minimum inhibitory concentration
MBC	minimum bactericidal concentration
LB	Lysogeny broth or Luria-Bertani broth
CFU	colony forming unit
OD	optical density
CLSI	clinical and laboratory standards institute
GM	glass microsphere
BDPA	bisdipicolylamine
FCC	flash column chromatography

CAMHB	cation-adjusted Mueller-Hinton broth
ATR	attenuated total reflectance
FTIR	fourier transform infrared spectroscopy
NMR	nuclear magnetic resonance
GFP	green fluorescent protein



LIST OF SYMBOLS

SYMBOLS

J	coupling constant
δ	chemical shift
μ	micro



CHAPTER 1

INTRODUCTION

Antibiotics have saved countless lives and have important roles in the medicinal area. Thanks to antibiotics, people do not lose their lives because of mere infections that would have resulted in the patient's death in the absence of antibiotics. However, due to misuse, overuse, and agricultural use of antibiotics, bacterial strains which are resistant to antibiotics in clinical use have emerged. Antibiotic resistance has been an important problem for decades. These resistant strains could be resistant to one antibiotic (i.e., single-resistant), or more than one antibiotic (i.e., multi-drug resistant). Antibiotic resistant bacterial strains are a significant threat to public health. Globally, millions of people lose their lives every year due to drug-resistant bacterial strains. Thus, there is a need to discover new antibiotics. Although new antibiotics have been introduced into clinical use, bacteria can gain resistance after some time. It is known that bacteria can gain resistance to antibiotics through gene transfer or genetic drift and exhibit resistance mechanisms such as limiting the uptake of antibiotics, modifying the targets of the antibiotics, inactivating the antibiotics, or efflux of antibiotics. Therefore, developing antibacterial strategies to which bacteria cannot gain resistance is essential. One approach is antimicrobial photodynamic therapy (aPDT), a system that generates singlet oxygen or reactive oxygen species with the help of photosensitizer dyes. Photosensitizers absorb energy from light and transfer the energy to the oxygen molecules to generate either singlet oxygen (Type II) or/and transfer the energy to the surrounding molecules to generate reactive oxygen species (Type I), which damage any living cells and result in cell death. Since aPDT does not discriminate between mammalian and bacterial cells,

with the help of the targeting groups to enable cellular level selectivity, aPDT could be an approach suitable for *in vivo* antibacterial inactivation.

1.1 General Properties of Bacteria

Bacteria are prokaryotic cells that do not have any nucleus or any other membrane-bound internal organelles. Most bacteria have sizes in the 1-10 μm range.¹ They are microscopic unicellular organisms that live in colonies comprised of massive numbers of cells. The activities of bacteria are regulated through communication within themselves and with their environment. Bacterial cells cover most of the biological population of the earth. Bacterial cells are essential for other living organisms. For example, they play an enormous role in the generation of O_2 .² Microbes (bacterial cells) are present in the food humans eat, the water humans consume, the infections that humans have, the soils that humans use for agriculture, and so on. Therefore, microbes and humans share the same biological area. Bacterial cells can survive harsh conditions. They can mutate and adapt to the conditions of the ecosystem in which they live.³ Since their life cycle is shorter than humans and other mammals, they evolve rather quickly than the other species. This provides them with an evolutionary advantage to survive harsh conditions.

The liquid or solid mixture that contains the required nutrients for bacterial cells to grow is called medium for singular and media for plural. Growth is used to refer to the multiplication of bacterial cells by mitosis. If a single cell of the bacteria is grown on the solid medium, it divides into millions of cells and the colony that thus forms becomes visible to the human eye. Liquid and solid media should consist of essential macronutrients for bacterial growth. Carbon and nitrogen are essential for every bacterial cell. Therefore, while preparing mediums, carbon and nitrogen sources such as sugars, nitrogen bases, fatty acids, organic acids, amino acids, and aromatic and other organic compounds are added to the mixture.

Bacterial cells are categorized into two main groups depending on the structures of the cell membrane, Gram (-) and Gram (+). The name came from a stain which is called Gram stain. The two types of bacteria are stained in either pink, classified as Gram (-), or purple, classified as Gram (+). The staining difference depends on the cell membrane of the bacterial cells. Gram (-) cells have a thin peptidoglycan layer that is surrounded by lipopolysaccharide, which creates an outer membrane. On the other hand, Gram (+) bacteria have layers of peptidoglycan as their cell membrane and lack an outer membrane. Therefore, Gram (+) bacteria have a cell membrane with a thickness of 30 –100 nanometers, whereas Gram (-) bacteria have a cell membrane with a thickness of a few nanometers.⁴⁵

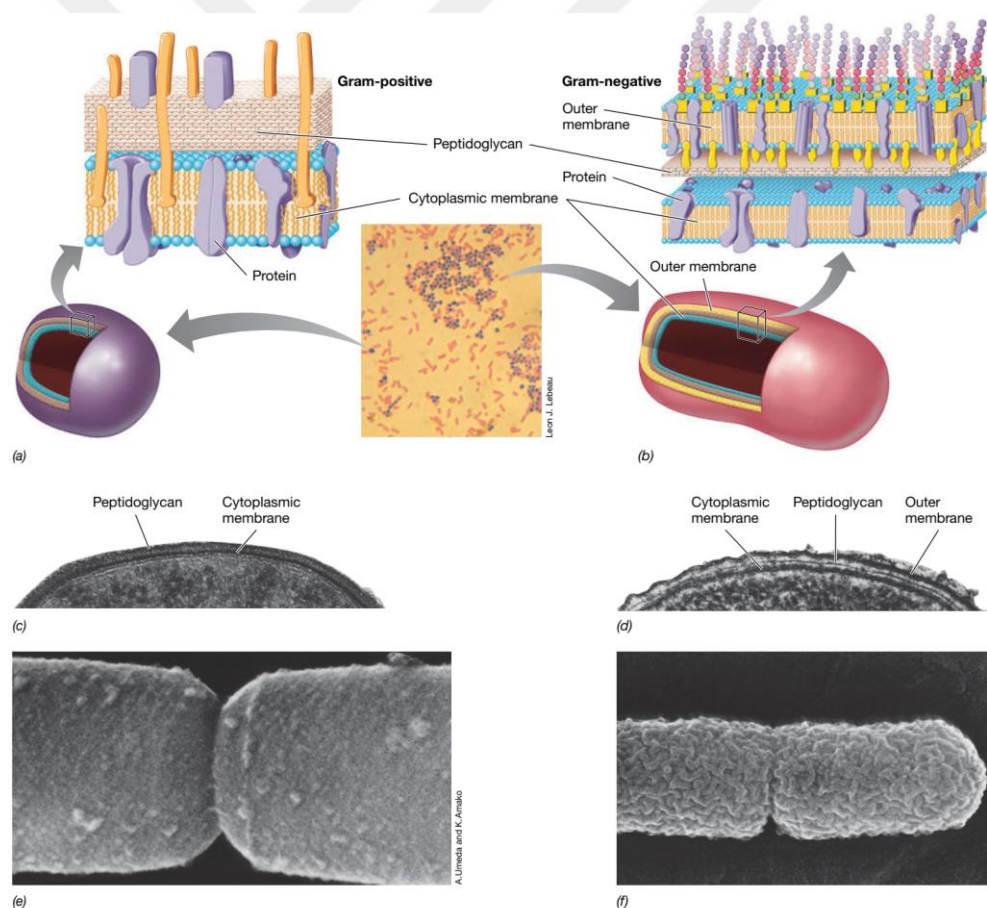


Figure 1. Gram (+) and Gram (-) cell membranes.¹

1.2 Antibiotic Resistance

Bacterial cells evolve and adapt much more quickly than humans since their life cycle is shorter. Therefore, when a stress source is present in the environment, they seek an opportunity to evolve to elude the stress source. People with infections are treated with antibiotics; if the subject misuses the antibiotic, meaning if they do not use the antibiotic for as long as required or at the dosage needed to kill all the bacteria (i.e., pathogens) that cause the infection, the remaining cells can evolve and find mechanisms to gain resistance to the used antibiotic.⁶ Moreover, the resistant bacteria can transfer their genes by horizontal gene transfer and make the whole colony resistant to the antibiotic. In a different case, if a person uses an antibiotic when they do not need to, the bacterial cells in human flora can gain resistance to the antibiotic they are exposed to.⁷ Moreover, when farm animals are given antibiotics to prevent diseases rather than treat them when they have the disease, bacterial cells in the animal flora can gain resistance, and humans can be exposed to the bacterial cells via animal products that they consume.

Antibiotic resistance mechanisms include the prevention of access to the target, changes in antibiotic targets by mutation,⁸ modification (and protection) of targets,⁹ and direct modification of antibiotics. Prevention of access can be either a reduction in membrane permeability or an increase in the efflux of the antibiotic. The other mechanism is change in the antibiotic target. Antibiotics work with a high selective affinity towards the antibiotic target. Bacteria can gain resistance through mutation in the antibiotic target side to prevent antibiotic binding without disturbing the function of the antibiotic target. As can be seen, there is more than one mechanism for gaining antibiotic resistance.¹⁰ The figure below (Figure 2) shows the chronology of antibiotics being introduced into clinical use (below the black line) and the chronology of the emergence of antibiotic resistance to them (upper side of the black line). This diagram demonstrates the fact that new antibiotics are in great need, and even if new antibiotics are commercially available, there will be a constant need for

new antibiotics since, over time, drug-resistant bacterial strains will continue to evolve.

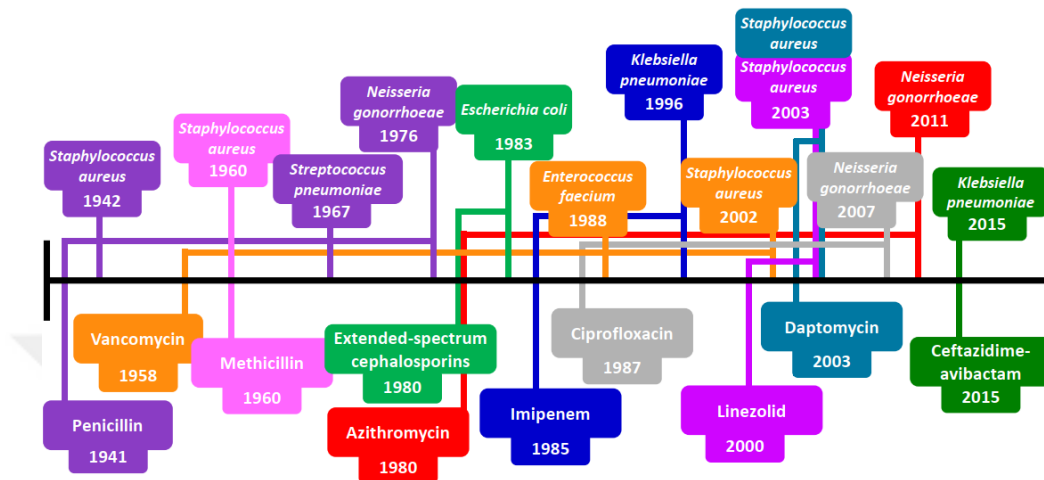


Figure 2. Chronology of antibiotics being introduced into clinical use (below the black line) and the chronology of the emergence of antibiotic resistance to those antibiotics (above the black line). Source: Centers for Disease Control and Prevention.

1.3 Differences Between Mammalian and Bacterial Cell Membranes

As mentioned before, bacterial cells have membranes composed of peptidoglycan and lipopolysaccharide.¹¹ Both Gram (-) and Gram (+) bacteria have negatively charged cell membranes.¹²⁻¹⁴ On the other hand, mammalian plasma membrane have an overall neutral charge with asymmetrically distributed negatively charged phosphatidylserine, mostly placed in the inner leaflet of the membrane. Therefore, one of the main differences between mammalian plasma membrane and bacteria cell membranes is that the former has neutral and the latter have negatively charged outer membranes, respectively. With this difference on hand, targeting bacteria in the presence of mammalian cells becomes possible.

1.4 Targeting Strategies

In order to achieve selective targeting of bacteria, therapeutic agents can be derivatized with different types of targeting groups. These groups include antimicrobial antibodies, peptides,^{15–17} glycodendrimers,¹⁸ antibiotics,¹⁹ and polycationic compounds and polymers.²⁰ Derivatizing therapeutic agents with targeting groups may enhance the therapeutic window by an increase in the effective dose of the agent and reducing systemic toxicity.^{21,22} For instance, derivatizing PDT agents with targeting groups may increase therapeutic window, because even though PDT agents are usually non-toxic to mammalian cells, after PDT treatment the subjects tend to become light sensitive and should wait in the dark until all the agent is out of the body.²³ If the PDT agent is derivatized with a targeting group, then all of the agents that are given would accumulate in the desired area, and only that part of the body becomes light-sensitive. For aPDT, targeting agents are generally antimicrobial antibodies, peptides, glycodendrimers, antibiotics, and polycationic compounds and polymers. One potential strategy for selective targeting is focusing on the difference between mammalian and bacterial cell membranes. The Smith Group has found out that zinc(II) bisdipicolylamine (i.e., Zn_2BDPA) complexes are able to selectively bind to negatively charged phosphate amphiphiles (Figure 3), which include phosphatidylglycerol, lipid A, and lipoteichoic acid (Figure 4)^{24,25} These negatively charged phosphate amphiphiles can be found on the surfaces of the membranes and walls of bacterial cells. Zn_2BDPA complexes are entirely synthetic, show low cytotoxicity to mammalian cells, are stable *in vivo*, and can be conjugated to different theranostic agents. These complexes can be used against all bacterial strains since they bind to cells regardless of the morphology or Gram-type of the cells. Zn_2BDPA conjugates have several application areas, which are agglutination,^{26,27} nuclear imaging,²⁸ and optical imaging of bacterial cells.^{29,30}

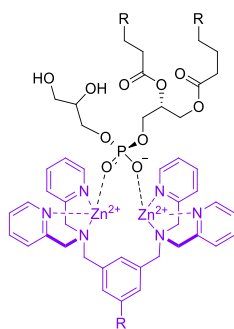


Figure 3. Interaction between a Zn_2BDPA complex and the negatively charged phospholipid, phosphatidylglycerol.

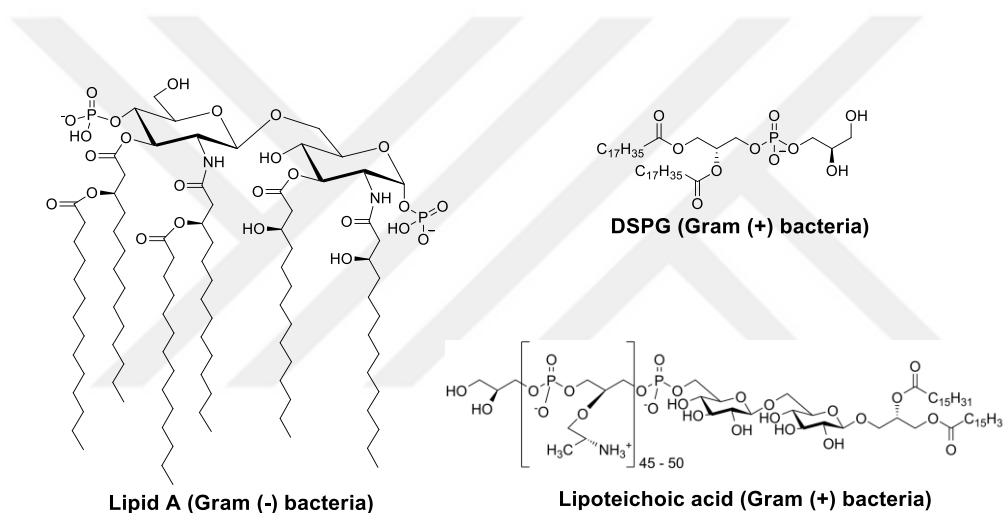


Figure 4. Structures of phosphatidylglycerol, lipid A, and lipoteichoic acid.³¹

1.5 Treatment of Antibiotic-Resistant Bacteria

Antibiotic-resistant bacteria are hard to treat, sometimes impossible with commercial drugs. When multi-drug resistant bacteria are the cause of an infection and if an antibiotic that is effective against these bacteria does not exist, then classical infection treatment will fail. For cases like this, there is a need for agents that bacteria cannot gain resistance against easily. One class of agents which will be focused on in this thesis is photodynamic therapy agents. Photodynamic therapy has great

potential for both clinical and experimental use. Recent experiments about photodynamic therapy on bacteria show no signs of emergence of resistance toward antimicrobial photodynamic therapy (aPDT).^{32–34}

1.6 Photodynamic Therapy

Photodynamic therapy (PDT) is a widely recognized method for treating various cancerous tumors. However, this technique is currently being expanded to treat several non-cancerous diseases. These diseases typically involve the excessive growth of undesired or abnormal cells, such as certain dermatological disorders, prostatic hyperplasia, and age-related macular degeneration.^{35,36} Also, there is an increased interest in the treatment of infections via PDT. PDT is centered on photosensitizers (PS), light, and oxygen and oxygen containing molecules. The PS, which is not toxic when it is not irradiated, is transformed to the excited state from its ground state by absorbing light. The excited state electron can go to the ground state by relaxation (fluorescence) or internal conversion. Also, the PS can undergo intersystem crossing to the triplet state, which is more stable and long-lived than the singlet state, by changing the excited electron spin. This electron in the triplet excited state can go to its ground state by phosphorescence or undergo two types of reactions. Photosensitizers can transfer the energy or electron to generate reactive oxygen species (ROS), either transferring energy to the oxygen molecules to generate singlet oxygen (Type II) or/and transferring an electron or hydrogen to the surrounding molecules to radicals such as peroxides, superoxide, or hydroxide radicals (Type I). Therefore, the relaxation of PS to the triplet state is essential to generating ROS. The ROS damages any living cells and results in cell death. Type I and Type II can occur simultaneously. Figure 5 shows the simplified Jablonski diagram demonstrating these relaxations, and Type I and II mechanisms.^{37–40}

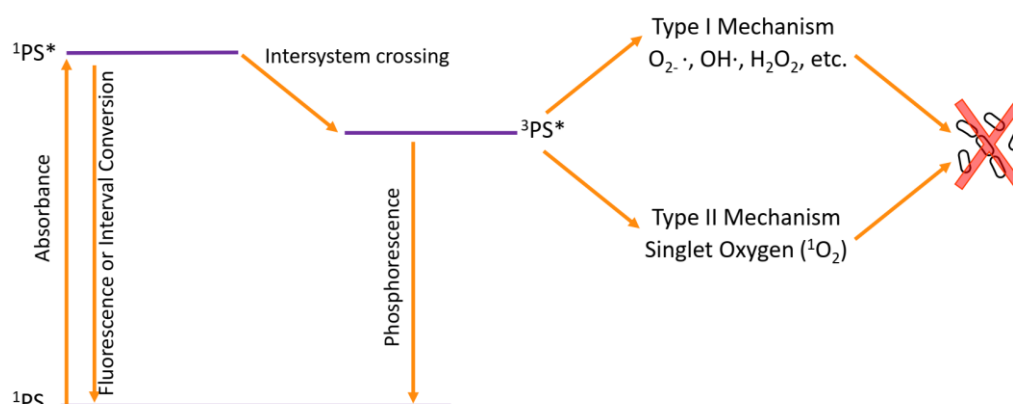


Figure 5. Simplified Jablonski Diagram³¹

Studies suggest that photodynamic therapy is particularly well-suited for treating localized microbial infections, including those that have become chronic after chemotherapy.^{41,42} PDT has several advantages, such as the development of treatment procedures that effectively target a wide range of microbial pathogens such as Gram (+) and Gram (-) bacteria, yeasts, fungi, and parasitic protozoa, and high susceptibility of antibiotic-resistant microbial strains to PDT, without the emergence of photoresistant species even after multiple treatments. Photodynamic therapy kills cells through oxidative stress; although bacteria can develop mechanisms to get through oxidative stress, the reactive nature of PS makes it highly unlikely to develop resistance against photodynamic therapy.⁴³ Also bacterial cells are usually not healthy enough to transfer genes after PDT treatment.⁴⁴ Another advantage of aPDT is the selective killing of microbial cells while sparing host tissues when the PDT agent is targeted. PDT can be easily applied for infections in externally accessible organs, such as skin, mouth, and so on.⁴⁵

In antimicrobial photodynamic therapy (aPDT or aPDI, antibacterial photodynamic inactivation), the photosensitizer is either inside the bacterial cells or at the cell membrane of the bacteria. When it is intended to be inside the bacterial cell, Gram (+) and Gram (-) strains behave differently. Gram (-) strains have less permeable

bacterial membranes, as discussed; thus, PS cannot go through the cell membrane as easily as against Gram (+) bacterial cells.^{46–48}

1.7 Porphyrins as PDT Agents

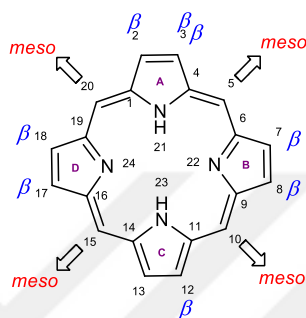


Figure 6. The general representation of a porphyrin molecule and its *meso* sites.

Porphyrins are a class of aromatic heterocyclic compounds found widely in nature. They play essential roles in vital biochemical processes such as oxygen transport (heme) and photosynthesis (chlorophylls). Porphyrin is an aromatic macrocyclic compound with 18π electrons. Due to their unique physicochemical properties, porphyrins have diverse applications in various fields. These applications include artificial photosynthesis, oxidation catalysis, sensors, nonlinear optics, nanomaterials, photodynamic therapy (PDT) for cancer treatment, and, more recently, the photoinactivation of microorganisms.^{49,50}

Different porphyrin derivatives are synthesized for each individual system that requires specific chemical, physical, and electronic properties. In the case of photoinactivation of microorganisms, porphyrin derivatives used as photosensitizers can be categorized into two groups. The first group consists of natural porphyrins and chemically modified versions of natural porphyrins. The second group exclusively includes synthetic porphyrins, which can be neutral, cationic, or anionic.⁵¹ According to Hückel's rule of aromaticity ($4n + 2 \pi$ electrons), the

porphyrin nucleus contains a total of 22 π electrons, with 18 π electrons being delocalized over the macrocycle. This aromatic site of porphyrins makes them susceptible to participate in electrophilic substitution reactions primarily at the *meso* positions, which are the most electron-dense and, consequently, the most reactive.⁵²

The synthetic photosensitizers (PS) used in antimicrobial photodynamic therapy (aPDT) are primarily based on *meso*-tetraarylporphyrins.⁵³ These porphyrins have gained popularity due to their simple synthesis and potential for further modification. Typically, the synthetic methods involve condensing pyrrole with suitable aldehydes in one-flask or two-step one-flask procedures.⁵⁴ The wide range of available aldehydes enables the introduction of different substituents at the *meso*-positions of the porphyrin ring. By controlling these substituents, a diverse group of porphyrins can be obtained, each with unique chemical, physical, and electronic properties suitable for specific applications.

Porphyrins exhibit highly intense absorption bands in the visible region of the electromagnetic spectrum due to the extensive electron delocalization within the macrocyclic structure. These absorption bands are responsible for the characteristic vibrant colors presented by porphyrins. Notably, porphyrins show a strong absorption band at around 420 nm, known as the Soret band. In addition, they exhibit weaker satellite absorption bands, known as Q bands, which are typically found between 600 nm and 800 nm.⁵⁵ Porphyrins have good singlet oxygen quantum yields, which is an essential characteristic of photosensitizers. The PDT applications of porphyrin are effective both in PDT and aPDT. Porphyrins are known to be non-toxic to the human body.^{37,56–60} Therefore, porphyrins are non-toxic (in the dark) and very efficient PDT agents. If porphyrin derivatives are coupled with Zn₂BDPA complexes, targeted PDT may be achieved.

The mechanism of porphyrin PDT is still under investigation and not completely understood. Many studies show that ROS from both type I and Type II mechanisms may cause cell damage by two main mechanisms.^{61–65} First is functional damage, which means inhibition of enzymes crucial to bacterial cells, oxidation of protein-

protein cross-links, and inhibition of metabolic processes such as DNA synthesis and glucose transport.^{66,67} The second one is cell membrane damage which is resulted when porphyrins are bound to the bacterial cell membrane and can lead to the breakdown of cellular contents and disruption of the membrane transport system and enzymes.^{68,69} While some damage to nucleic acids may occur as a result of porphyrin exposure, it has been observed that these damages can be repaired by DNA repair systems.⁷⁰

1.8 Heavy Atom Effect

The increase in singlet oxygen ($^1\text{O}_2$) generation caused by heavy atoms or substances containing heavy atoms is known as the heavy atom effect. The quantum yield of singlet oxygen, a measure of singlet oxygen generating efficiency, may be strongly influenced by the heavy atom effect.⁷¹

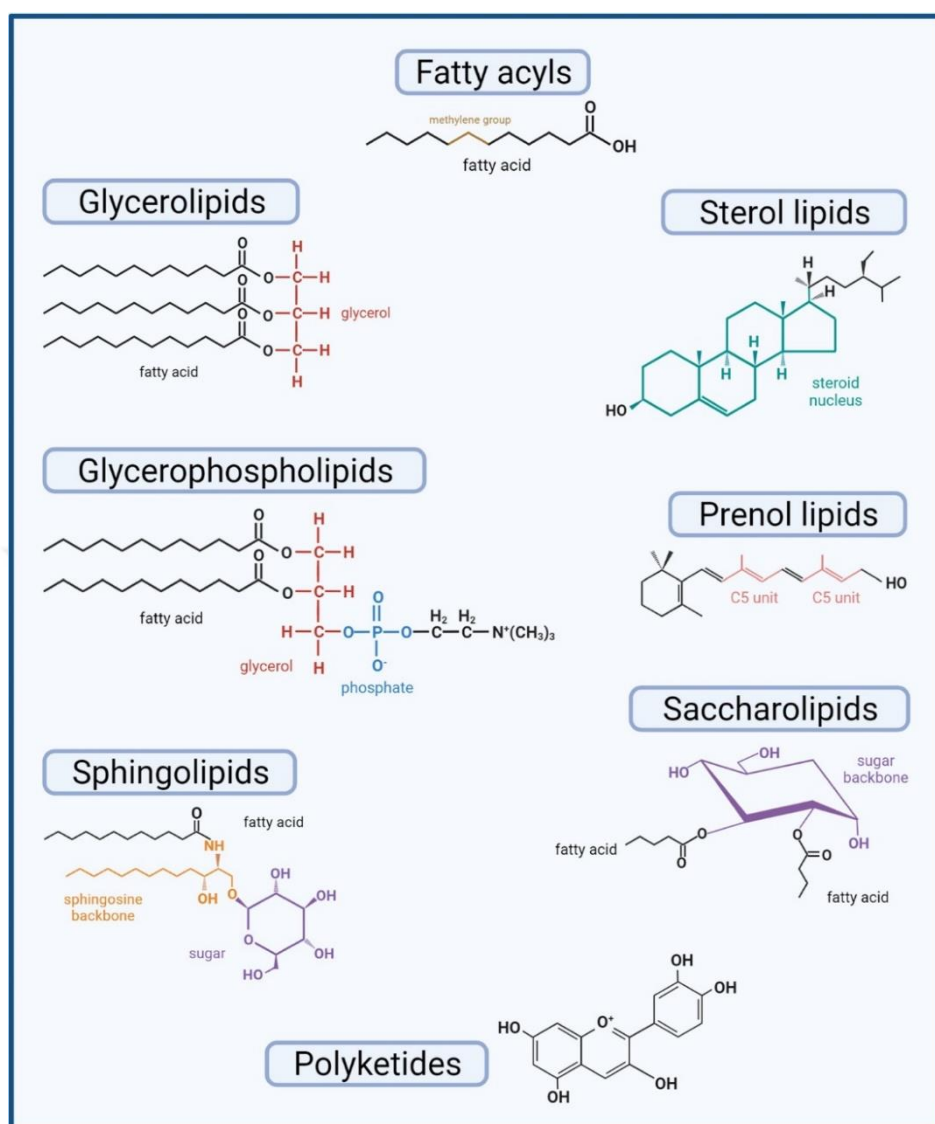
What causes the heavy atom effect is the heavy atom's capacity to promote intersystem crossing (ISC) from the excited singlet state to the triplet state. Then, the triplet state population, which is made possible by the ISC process, interacts with ground state molecular oxygen ($^3\text{O}_2$) to produce singlet oxygen ($^1\text{O}_2$). The presence of heavy atoms permits ISC by promoting spin-orbit coupling, which is the interaction between the spin of an electron and its orbital motion influenced by the heavy atom's strong nuclear charge. This coupling leads to faster conversion of the singlet state to the triplet state, increasing the likelihood of singlet oxygen generation. Iodine (I), bromine (Br), chlorine (Cl), and heavy metal ions, including platinum (Pt), palladium (Pd), and mercury (Hg) are a few frequent heavy atoms that show the heavy atom effect. The exact mechanism of the heavy atom effect on singlet oxygen quantum yield is complex and depends on factors such as the nature of the heavy atom, its proximity to the photosensitizer, and the specific electronic and molecular orbital interactions involved. The heavy atom effect is important in the case of PDT, in which Type II mechanism depend on singlet oxygen quantum yield.^{72–76}

1.9 Liposomes

1.9.1 Lipids

Fatty acyl groups are essential components of complex lipids and play crucial roles in various biological processes. They serve as the primary building blocks for lipids and are characterized by a hydrophobic nature derived from a chain of methylene groups. Fatty acids can be further classified based on the presence of double bonds in their hydrocarbon chains. Examples of fatty acid esters include wax monoesters and diesters. These molecules exhibit diverse functions, including energy storage, insulation, and structural support. Fatty acids are not typically known to form liposomes on their own. However, they can contribute to the formation and stability of lipid structures such as liposomes when incorporated into glycerophospholipids or sphingolipids.

Glycerolipids are major constituents of cellular membranes (except triacylglycerols) and are primarily composed of mono-, di-, and trisubstituted glycerols. The most well-known glycerolipids are acylglycerols, which are formed by esterification of fatty acids with glycerol. Acylglycerols serve as a major energy reserve in the form of triglycerides and are stored in adipose tissue. They play critical roles in lipid metabolism, membrane integrity, and signaling processes. Glycerolipids, such as triglycerides, do not readily form liposomes or micelles due to their bulky hydrophobic tails and limited hydrophilic regions. Their primary role is energy storage rather than self-assembly into organized structures like liposomes or micelles. Glycerolipids are typically present in the form of lipid droplets within cells.



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Figure 7. Different classes of lipids. ⁷⁷

Glycerophospholipids, also known as phospholipids, are crucial components of cellular membranes. They possess a glycerol backbone, a phosphate group, and two fatty acid chains. The unique amphipathic structure of glycerophospholipids allows them to form lipid bilayers in membranes, providing a barrier and compartmentalization for cellular processes. These lipids are involved in cell signaling, membrane fluidity regulation, and the recruitment of proteins to specific membrane regions. Glycerophospholipids, such as phosphatidylcholine and

phosphatidylserine, can self-assemble into liposomes in aqueous solutions. The hydrophobic tails of the lipids align together to form a bilayer, while the hydrophilic head groups face the surrounding aqueous environment. Liposomes can vary in size and can encapsulate aqueous substances within their internal compartments.

Sphingolipids constitute a diverse family of lipids that share a common sphingoid based backbone synthesized from serine and a long-chain fatty acyl-CoA. This backbone can be further modified to form various sphingolipid derivatives, including ceramides, sphingomyelins, glycosphingolipids, and gangliosides. Sphingolipids play critical roles in cellular signaling, membrane stability, cell recognition, and intracellular trafficking. Sphingolipids, including sphingomyelins and ceramides, also have the ability to form liposomes or micelles. The structure of sphingolipids is similar to that of glycerophospholipids, with a hydrophobic tail and a polar head group. Depending on the conditions and the presence of other molecules, sphingolipids can form liposomes or adopt a micellar structure.

Sterol lipids are important constituents of cell membranes and exhibit diverse biological functions. Cholesterol is the most well-known sterol lipid found in animals, while phytosterols are prominent in plants. Sterol lipids are involved in membrane fluidity regulation, cell signaling, and the formation of specialized membrane microdomains called lipid rafts. Additionally, sterols serve as precursors for the synthesis of steroid hormones and bile acids. Sterol lipids, such as cholesterol in animal cells and phytosterols in plant cells, do not form liposomes or micelles by themselves. However, they play a critical role in modifying the fluidity and permeability of lipid bilayers. Cholesterol, for example, helps regulate membrane rigidity and stability by inserting itself between phospholipid molecules. This property of sterol lipids influences the behavior and properties of liposomes and micelles that contain other lipid components.

Prenol lipids are derived from isopentenyl diphosphate and dimethyl diphosphate, which are produced through the mevalonic acid pathway. Prenol lipids include compounds such as ubiquinones, dolichols, and isoprenoids. They play essential

roles in cellular processes, including energy transfer, electron transport, and the synthesis of molecules such as vitamins and hormones. Prenol lipids, including carotenoids, are not directly involved in the formation of liposomes or micelles. Their primary functions are related to signaling, pigmentation, and antioxidant activity. Prenol lipids can be incorporated into lipid-based systems for drug delivery, but liposomes or micelles serve as the carriers rather than the preno lipids themselves.

Saccharolipids are unique lipids that consist of a fatty acid linked to a sugar backbone. They are highly abundant in lipopolysaccharides of Gram (-) bacteria and contribute to the structural integrity of bacterial membranes. Saccharolipids play important roles in host-pathogen interactions, bacterial virulence, and immune responses. Saccharolipids do not typically form liposomes or micelles.⁷⁸⁻⁸¹

1.9.2 Micelles and Bilayers

Amphiphilic molecules, which have both hydrophilic and hydrophobic segments, exhibit distinctive self-assembly behavior in aqueous solutions. Their ability to form aggregates, notably micelles, depends on the conditions and concentration of the system. Once the concentration of amphiphilic molecules exceeds a critical threshold known as the critical micelle concentration (CMC), the process of self-assembly begins. When that happens, the hydrophobic moieties of the molecules assemble, effecting an arrangement that shields them from the surrounding aqueous solvent while the hydrophilic portions remain exposed. This spontaneous arrangement results in the formation of micellar colloids, involving many individual molecules organized into spherical structures. Micelles are fundamentally dynamic particles, repeatedly exchanging monomeric constituents with the surrounding solvent. They exist in a state of equilibrium in which the formation and dissociation of micelles happen simultaneously. This dynamic system makes micelles versatile in many biological and physicochemical processes. A comprehensive understanding of the factors leading to micelle formation, including temperature, solvent composition,

and molecular structure, is known. Micelles are used in various fields, such as drug delivery, surface modification, and nanoparticle synthesis.⁸²

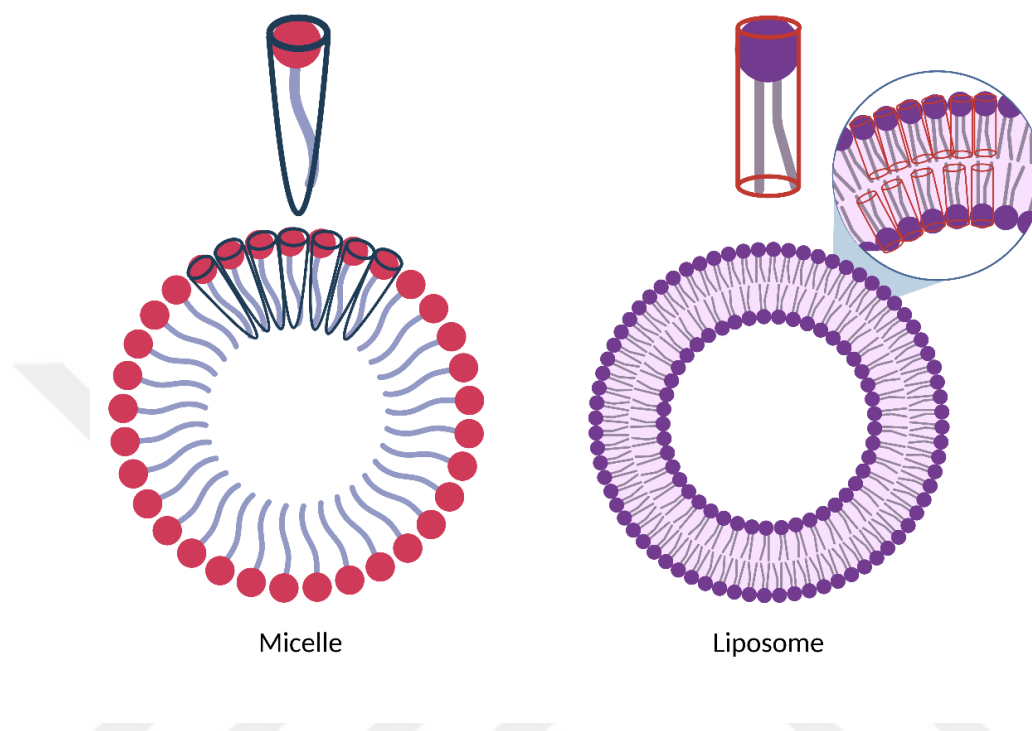


Figure 8. Structures of Micelles and Liposomes.

Liposomes, as self-assembled structures, are spherical vesicles composed of lipid bilayers that are constructed from specific types of amphiphilic lipids. These lipids consist of phospholipids, glycolipids, and cholesterol, which contribute to the formation and stability of liposomes and bilayers. Phospholipids, the most common lipid components of liposomes and bilayers, have a hydrophilic head group and two hydrophobic fatty acid tails. The interaction of attractive forces between the hydrophilic and hydrophobic regions allows phospholipids to arrange themselves into bilayers spontaneously. In this arrangement, the hydrophilic heads face the surrounding aqueous environment, while the hydrophobic tails remain isolated in the interior side of the bilayer. The amphiphilic nature of these lipids enables them to undergo self-assembly, facilitating the formation of liposomes and bilayers. These

structures serve as encapsulating systems, creating aqueous compartments enclosed within the hydrophobic core of the lipid bilayer.

Liposomes and micelles are self-assembled structures formed by amphiphilic molecules. Liposomes are spherical vesicles with multiple lipid bilayers that surround an aqueous center. In contrast, micelles are smaller, single-layered structures without separate compartments. Liposomes have a more complex structure and can vary in size, while micelles are simpler and typically smaller. Differences lie in their size, organization, and the presence of distinct compartments.⁸³

1.9.3 Liposomal Encapsulation

Drug delivery systems are being developed with the aim to improve the therapeutic index of drugs by increasing their concentration at the target site, extending their presence in the target cells, and reducing side effects.⁸⁴ Most of the recently developed targeted drug delivery systems use nano vehicles to deliver the active drug to the target site, which enhances the drug's pharmacological properties and overcomes its undesirable characteristics, such as side effects, by improving drug pharmacokinetics, biodistribution, and acting as drug reservoirs.^{85,86} Nanoparticles (NPs) are typically utilized in these systems and can range in size from a few nanometers to several hundred nanometers, depending on the specific application. Various natural, organic, and inorganic materials, such as ceramics, polymers, metals, and lipids, including micelles and liposomes, are employed to create these nanoparticles. Many innovative drug delivery systems, including liposomal drug delivery systems, have been developed to take over the limitations related to the lack of selectivity and stability observed in conventional cytostatic drugs. Liposomes, as mentioned before, are self-assembling structures consisting of lipid bilayers. They are a sophisticated and adaptable technology for targeted drug delivery.^{87,88}

Liposome preparation and lipid formulation choices highly depend on the desired surface charge, size, and physical and chemical properties.⁸⁹ Liposome preparation can be done in three different ways: mechanical dispersion, solvent dispersion, and detergent removal. Mechanical dispersion often means sonication, extrusion, freeze-thaw, utilization of a pressure cell, micro-emulsification, membrane extrusion, and so on.⁹⁰ Solvent dispersion methods are either injection and reverse phase evaporation. Lastly, the detergent removal technique is another method to prepare liposomes, but it bears the unfortunate possibility of the detergent not being fully removed from liposomes.^{91,92}

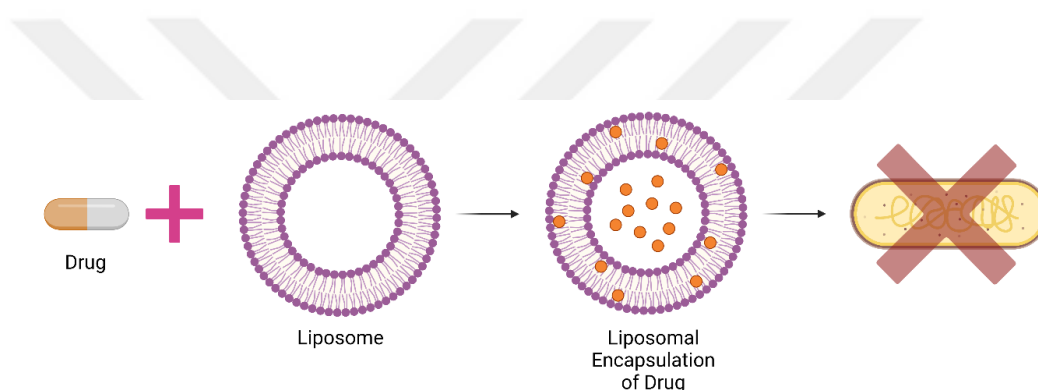


Figure 9. Schematic Scheme of Liposomal Encapsulation of drugs.

Liposomes can be categorized by their structures into four categories: unilamellar vesicles (SUV), large unilamellar vesicles (LUV), multilamellar vesicle (MLV), and multivesicular vesicles (MVV). In a unilamellar structure, liposomes have a single phospholipid bilayer. Oppositely, MLVs exhibit an onion-like structure with multiple concentric phospholipid bilayers, and MVVs consist of multiple unilamellar vesicles formed within larger liposomes. Unilamellar vesicles generally have diameters ranging from 50 to 250 nm, except Giant Unilamellar vesicles which can be several microns in diameter and are well-suited for encapsulating water-soluble drugs since they have a big aqueous core. On the other hand, multilamellar vesicles have larger diameters, typically ranging from 1 to 5 μm .⁹³

Therapeutic drugs are typically incorporated into liposomes through various physical interactions, such as entrapment, surface attachment, or encapsulation.⁹⁴ These methods involve the drug being physically trapped in the liposome structure, attached to the surface, or encapsulated in liposomes. The encapsulation efficiency differs for different sizes of liposomes and increases with increasing size.⁹⁵

Encapsulation of photosensitizers within liposomes also offers several advantages, including reduced side effects and improved solubility.⁹⁶ For instance, hydrophobic photosensitizers like porphyrins, which have low solubility in aqueous systems such as biological environments, can have significantly increased solubility when encapsulated in liposomes.⁹⁷ This enhanced solubility enables their effective delivery in aqueous solutions. Furthermore, encapsulation prevents the aggregation of porphyrins by inhibiting their stacking, which can negatively impact their therapeutic efficacy.⁹⁸ As a result, porphyrins encapsulated in liposomes exhibit improved effectiveness in photodynamic therapy.⁹⁹ Liposome encapsulation of photosensitizers can also contribute to targeted drug delivery systems, allowing for specific targeting and localized treatment. Liposomes are an effective tool for improving the stability of formulations when used for systemic delivery. By encapsulating photosensitizers within liposomes, the stability of the photosensitizer formulation is enhanced. This encapsulation provides protection to the photosensitizer, preventing it from being metabolized or inactivated in the plasma during circulation within the bloodstream. The liposomal structure acts as a protective barrier, shielding the encapsulated photosensitizer and allowing it to retain its activity until it reaches the target site. This increased stability ensures that the photosensitizer remains intact and active, enhancing its therapeutic efficacy during systemic delivery.

1.10 Application on Bacteria

1.10.1 Bacteria Handling

Microorganisms need a series of nutrients to grow, and when they are grown in the laboratory, it is important to provide microorganisms with all the nutrients they need. The medium prepared for bacterial growth is called growth medium. Ingredients of growth medium differ from microorganisms that are desired to culture. However, the major ingredients are water, carbon, oxygen, nitrogen, phosphorus, and sulfur for any desired growth medium. Water content is about 90% of the total weight. The fundamental ingredients are protein hydrolysates, in other words, peptones, meat extracts, malt extracts, yeast extracts, agar, bile salts, carbohydrates, minerals, and chelating agents. For example, LB (lysogeny broth or Luria–Bertani medium) consists of yeast extract, peptone, and sodium chloride. The overnight inoculum of bacterial cells to be harvested in the experiment is generally incubated in a liquid growth medium. On the other hand, sometimes a solid growth medium is desired, and therefore agar is added to the medium.

The unit used to specify the number of bacteria in a liquid medium is CFU/mL, which means colony-forming unit per mL. Since the pH of the medium is important for bacterial cells, they are generally in a buffer solution. Bacterial cell number can be associated with an optical density (OD) at a specific wavelength, generally 600 nm. The relationship between OD and bacterial cell number is determined by colony numbers on a solid growth medium after serial dilutions. Bacterial suspensions are serially diluted to 10^3 CFU/mL, so that they contain 25 bacteria in 25 μ L. Thus, when 25 μ L is plated on the LB-agar, the expected colony number is 25. If not, the relationship between OD and colony number is calculated, and the experiment is repeated until desired colony number on the agar plate is achieved. After this relationship is established, all calculations related to the experiment can be done using that relationship.

The measurement of absorbance acts as an indicator of bacterial growth, as it quantifies the optical density of a bacterial suspension. An increase in absorbance over time implies the multiplication of bacterial cells. the Minimum Inhibitory Concentration (MIC) is defined as the minimum concentration in which there is no bacterial growth. To determine the MIC of antibiotics, the broth microdilution procedure is used. To conduct broth microdilution, a 96-well plate is used, wherein different concentrations of the antibiotic agents under investigation are distributed. The concentration gradient decreases progressively from left to right within each row. Furthermore, each row can contain a distinct antibiotic agent of interest, enabling the evaluation of multiple agents in a single experiment. To ensure reliable and reproducible results, it is recommended by the Clinical and Laboratory Standards Institute (CLSI) that each well contains a minimum volume of 100 μ L. Additionally, the preferred broth medium for broth microdilution is Cation-adjusted Mueller-Hinton Broth, which provides an optimal growth environment for bacterial cultures. The bacterial cell density in each well should be approximately 5×10^5 CFU/mL. Following the addition of broth, antibiotic agents, and bacteria to the well-plate, the plate then incubated at a temperature of 35 ± 2 °C. The determination of MIC can be carried out through visual inspection, where the lowest concentration of the antibiotic that demonstrates no visible growth of bacteria is considered the MIC. Alternatively, plate readers can be employed to measure optical density or turbidity, providing a quantitative assessment of bacterial growth inhibition and MIC determination. However, it is important to acknowledge that the absence of growth, no absorbance in the cell, does not necessarily imply bacterial cell death. Bacteria can enter a state of rest or non-multiplication. They would remain metabolically active but do not multiply. This is known as bacterial viability without growth. Therefore, to determine the viability and death of bacterial cells, methodologies such as colony counting or streaking should be employed. If there isn't any growth in a solid medium such as LB-Agar, then that concentration should be reported as Minimum Bactericidal Concentration (MBC), the minimum concentration at which complete bacterial cell death is observed.

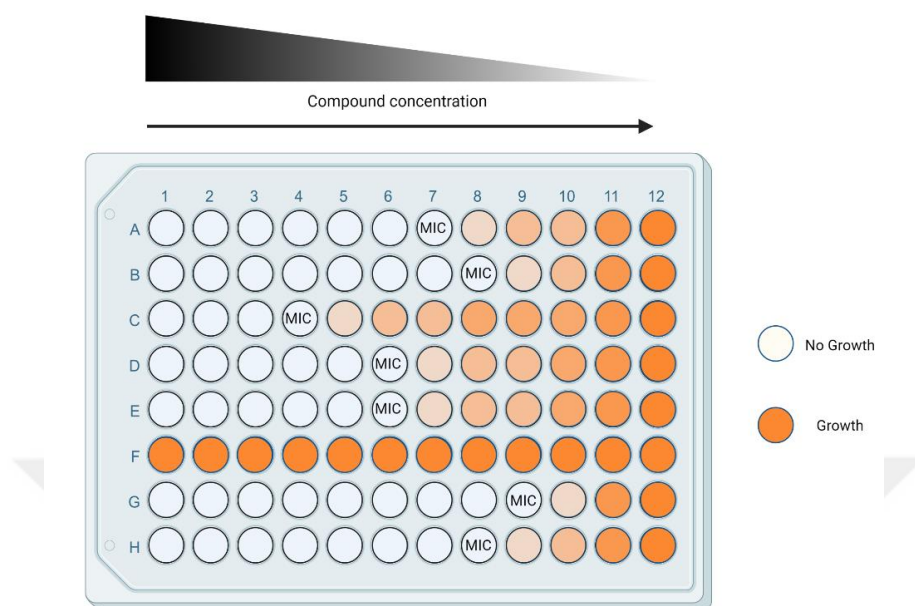


Figure 10. Schematic diagram of a 96 well-plate after Broth Microdilution experiment.

1.11 Bacteremia

Bacteremia is defined as the presence of bacterial cells in the blood, which otherwise is sterile. People can get infected after oral operations or after minor surgeries. A healthy human body can defend itself even when it is not treated. However, when the immune system is not at its best or is overwhelmed, the presence of bacteria in the blood can cause a series of health complications. For example, untreated bacteremia can result in sepsis, septic shock, systemic inflammatory response syndrome (SIRS), and multiple organ failure.^{100,101} When hospitalized people are investigated, the common sources of infections are found to be catheters and tubes inserted into the respiratory tract. The most common Gram (-) bacteria is found to be *Escherichia coli*, and the most common Gram (+) bacteria in blood infections is *Staphylococcus aureus*.^{102–104} In 2017, around 48.9 million incidents of sepsis were documented on a global scale, with approximately 11.0 million deaths attributed to

sepsis. These sepsis-related fatalities accounted for approximately 19.7% of all deaths worldwide.¹⁰⁵ Therefore, immediate detection and identification of the pathogen of infected blood samples are considered among the most crucial responsibilities of the microbiology laboratory.¹⁰⁶ Bacterial cells in the blood are often times low in number, such as <1-10 CFU (colony forming unit)/mL. Thus, a larger amount of blood samples for culturing is needed, samples are frequently 20-30 mL range.^{107,108} Antibodies, phagocytes, complement, and antimicrobial agents are known to be the factors that can hinder the isolation of microorganisms from blood samples. Various possible solutions to this problem have been proposed. For example, one was to dilute blood samples in broth at a ratio of 1:15-1:20 to neutralize serum bactericidal activity.¹⁰⁹ The other one is incorporating sodium polyanetholsulfonate (SPS) at a concentration of 0.025% in blood culture media to inhibit phagocytosis, complement, and lysozyme. However, even with the inclusion of SPS, it is believed that a blood-to-broth ratio of at least 1:10 is optimal for achieving favorable results.¹¹⁰

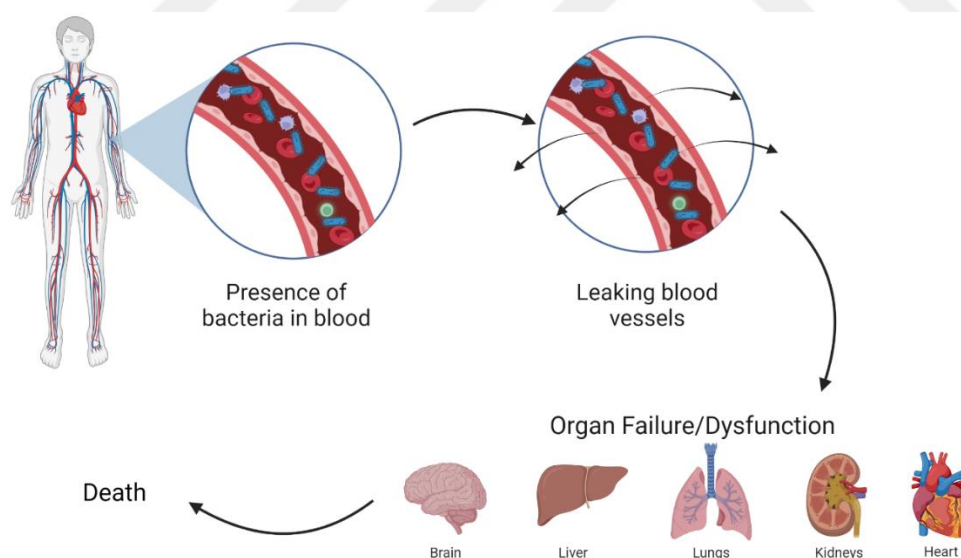


Figure 11. Schematic summary of bacteremia and potential consequences.

For all of these aforementioned reasons, blood culture in the laboratory can take up to 24 hours, which is crucial for patients. To speed up the process, bacterial

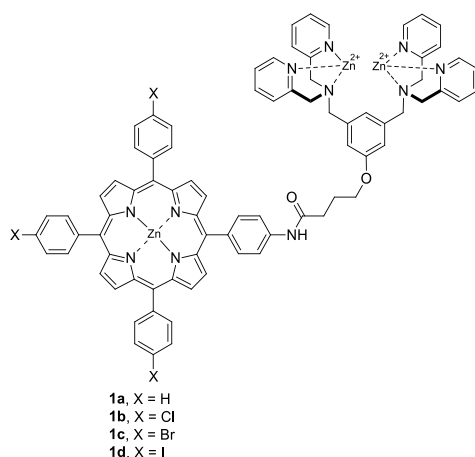
sequestration can be a solution. For example, Zn₂BDPA-bearing cellulose microspheres are known to bind bacteria and can be used in bacterial sequestration.¹¹¹ Cellulose microspheres should be larger than 50 µm for whole blood sequestration to prevent clumping of red blood cells, white blood cells, and platelets during filtration.^{112,113}

Glass microspheres bearing the Zn₂BDPA complex could also be used for whole blood filtration for bacterial sequestration. Since glass microspheres are smoother than cellulose microspheres, adsorption will be less, which means better whole blood filtration. Glass microspheres are easier to derivatize than cellulose microspheres since they do not have layers as cellulose microspheres have.

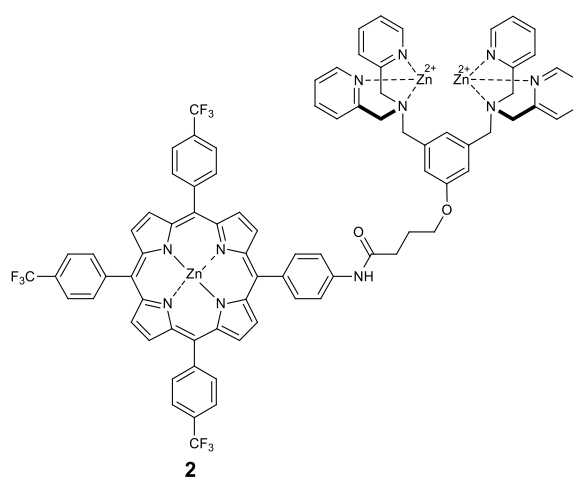
1.12 Objectives of These Studies

This thesis describes experimental work done towards the development of methods for the evaluation of antibacterial photodynamic therapy (aPDT) agents, liposomal encapsulation of aPDT agents and antibiotics, and glass microspheres which may sequester bacterial cells from aqueous media. Work was done on the following specific four areas:

- i. The aim of the first experimental part of this thesis was to develop methods for the evaluation PDT systems that were designed to exhibit antimicrobial photodynamic activity towards Gram (+) and Gram (-) bacterial strains, specifically porphyrin derivatives conjugated to Zn₂BDPA complexes (**1a-d**, see structure below) which are known to bind to bacterial cell membranes. **1d** was selected in these studies as it was thought that the presence of iodine substituents would render these the most efficient photosensitizer in this set since the iodine substituent increases singlet oxygen quantum yields due to the heavy atom effect.

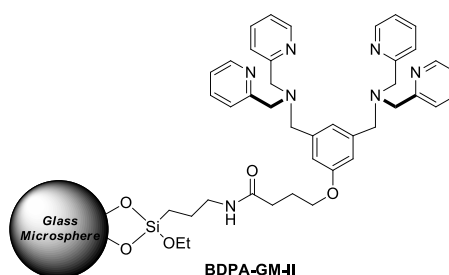
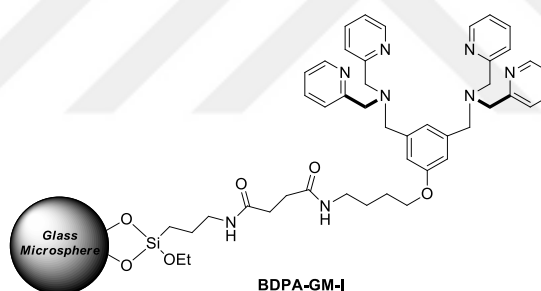


- ii. The second set of experiments aimed the liposomal encapsulation of targeted porphyrin derivatives and investigation of the aPDT potential of these liposomal systems. It was postulated that bacterial inactivation would increase with the liposomal encapsulation of porphyrins. To this end, liposomal encapsulation of targeted porphyrin derivative **2** (see structure below) was aimed. **2** and Liposomal-**2** were applied to bacterial strains as photosensitizers to determine whether liposomal encapsulation had any positive effect. Targeted porphyrin derivative **2** was chosen to be encapsulated because the $-\text{CF}_3$ groups at the *meso* positions were believed to increase interaction between the PDT agent and the lipids of the liposomes, and as a result encapsulation would be more efficient.



- iii. Liposomal encapsulation is also known to increase the efficacy of antibiotics. Therefore, in this part of the thesis, the aim was to encapsulate antibiotic rifampicin within the liposomes bearing Zn_2BDPA complexes on their outer surfaces.

- iv. The last part of this thesis summarizes studies towards the development of glass microspheres (i.e., GMs) derivatized with Zn_2BDPA complexes (i.e., BDPA-GM-I and BDPA-GM-II, see structures below) for the sequestration of bacterial cells from aqueous liquids, blood in particular. The ultimate objective is to develop glass microspheres like these for removal of bacterial cells from the blood of bacteremia patients and facilitate the characterization of bacterial strains that may be present in the bloodstream





CHAPTER 2

EXPERIMENTAL

2.1 General Information

The proton (^1H NMR) nuclear resonance spectra were acquired using Bruker Spectrospin Avance DPX-400 MHz spectrometers operating at 400 MHz. The chemical shifts are reported in parts per million (ppm) on the delta scale (δ). The solvent peak, CDCl_3 , was utilized as the reference value with a chemical shift of 7.27 ppm. The proton data notation includes the following abbreviations: s for singlet, d for doublet, t for triplet, q for quartet, dd for doublet of doublets, dq for doublet of quartets, m for multiplet, b for broad, and app for apparent. E. Merck pre-coated aluminum-backed silica gel 60F-254 plates were utilized for analytical TLC analysis. The TLC plates were visualized under UV light (254 nm) or with appropriate stains. Flash column chromatography (FCC) was carried out using SiliCycle SiliaFlash silica gel (40 - 63 μm , 60 Å), following the procedure developed by Still and colleagues.¹¹⁴ Solvents employed for chromatography or reactions underwent a drying and purification process to ensure purity. Ethyl acetate (EtOAc) was distilled from CaCl_2 , while hexanes were distilled from CaCl_2 . Reagent-grade methylene chloride (CH_2Cl_2) and chloroform (CHCl_3) were distilled from CaH_2 before use in reactions. Distilled methylene chloride (CaCl_2) and analysis-grade chloroform were used for chromatography purposes. Pre-dried dimethylformamide (DMF) and tetrahydrofuran (THF) were employed for reactions. In both chromatography and reactions, ACS-grade methanol and ethanol were used. Shimadzu UV-1700 UV-Vis Pharmaspec Spectrophotometer was used to collect absorbance spectra. Thermo Scientific Nicolet™ iS™ 10 FTIR Spectrometer was used to collect (Attenuated total reflection) ATR spectra. DLS data and zeta potentials were recorded with Zetasizer

Nano-ZS. Confocal microscopy images were recorded using Zeiss LSM 800 Confocal Microscope at 63x magnification.

Staphylococcus aureus ATCC 25923, *Bacillus cereus* ATCC 14579, *Escherichia coli* Top 10, *Pseudomonas aeruginosa* ATCC 9027, GFP-expressing *Escherichia coli* K12 pSB1C3-GFP, and *Bacillus subtilis* ATCC 6633 were received as gifts. *Escherichia coli* ATCC 25922 was purchased from KWIK-STIK. *Pseudomonas aeruginosa* ATCC 27853 was purchased from Public Health General Directorate of Turkey.

All other chemicals were procured from reputable sources and used as received. Glass microspheres were purchased from Swarco as “Swarcoblast Glass Blasting Beads” 150-250 μm , Soda-Lime Glass, chemical composition is SiO_2 68.0–75.0%, Al_2O_3 0–2.5%, MgO 0–5.0%, CaO 7.0–12.0%, Na_2O 12.0–18.0%, other max. 2.0%.

2.2 Bacterial Inactivation Experiments

Bacterial Strains: The Gram (+) bacterial strains used in this section were *Staphylococcus aureus* ATCC 25923 and *Bacillus cereus* ATCC 14579. The Gram (-) bacterial strains were *Escherichia coli* Top 10, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 9027.

2.2.1 Preparation of Bacterial Suspensions for all Experiments

To prepare the bacterial suspensions, the following steps were followed:

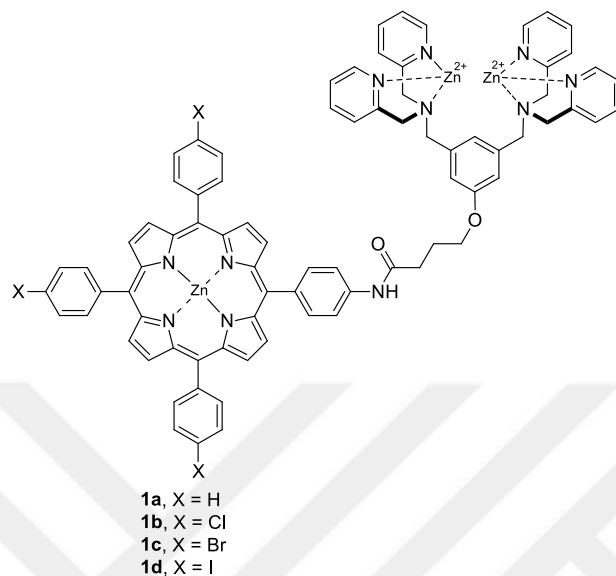
A 5 mL sample of sterile Lysogeny broth (LB) was inoculated with frozen bacterial stock suspensions, and the broth cultures were grown in a shaking incubator at 37 °C and 200 rpm for 16-18 hours. After the incubation, the bacterial cells were pelleted through centrifugation. The centrifugation was performed at 4000 rpm (1180 g) for 2 minutes for all the bacterial strains. However, for *Pseudomonas aeruginosa*, the

centrifugation speed was increased to 6000 rpm (2656 g) for 2 minutes. The pelleted bacterial cells were resuspended in 1 mL of HEPES pH 7.4 buffer. The suspension was centrifuged again using the same parameters as before, and the supernatant was removed. The washing procedure was repeated three more times, with each round involving resuspending the bacterial pellets in 1 mL of HEPES pH 7.4 buffer and centrifuging to remove the supernatant. Finally, the pelleted bacterial cells were resuspended in 1 mL of HEPES pH 7.4 buffer. To adjust the bacterial cell number, the optical densities (OD) of the washed suspensions were measured at 600 nm. The suspensions were adjusted to a concentration of 10^8 CFU/mL (colony forming units) by diluting the suspensions based on their OD values. By following these steps, bacterial suspensions with the desired concentration and in the appropriate buffer were prepared for further experiments or analysis.

2.2.2 Light Characterization for Photodynamic Inactivation Experiments

In bacterial photoinactivation studies, a 400 W halogen bulb was used as the light source. The light emitted from the bulb was filtered to ensure specific characteristics. Firstly, a cooling filter made of plexiglass was used, and cold water was circulated through it to prevent overheating. Secondly, a green filter was applied to remove all emissions below 400 nm. This filtration process helped to achieve the desired wavelength range for effective bacterial photoinactivation. During the experiments, the bacterial suspensions were positioned at a distance of 25 cm from the light source. This distance ensured that the desired fluence of 34 mW/cm^2 was delivered to the bacterial samples. The experiments were conducted at room temperature, meaning the bacterial suspensions were maintained at the ambient temperature of the laboratory during the photoinactivation process.

2.2.3 First Generation Approach for Preparation of Bacterial Suspensions for Irradiation and for Dark Treatment



To prepare the bacterial suspensions for photoinactivation experiments, the following steps were followed:

In a sterile 2 mL Eppendorf tube, an appropriate volume from 10 mM HEPES pH 7.4 buffer was added so that the final volume would be 1500 μL at the end. 3 μL of 10 mM $\text{Zn}(\text{NO}_3)_2$ was added so that the system contained excess Zn^{2+} ions and the stability of the Zn_2BDPA complex was assured upon dilution. For every desired final concentration of porphyrin, serial dilution was performed to obtain 100 times greater than desired final concentration. 15 μL of thus prepared stock solutions of Zn_2BDPA -porphyrin derivative **1d**, synthesized by another student¹¹⁵, in DMSO was added to the Eppendorf tube. Then, 75 μL of a bacterial suspension with a bacterial cell number of 10^8 CFU/mL was added to the tube. Thus, the final bacterial suspensions

had a concentration of 5×10^6 CFU/mL. The final concentration of the photosensitizer in the suspensions ranged from 1-20 μ M (Figure 12).

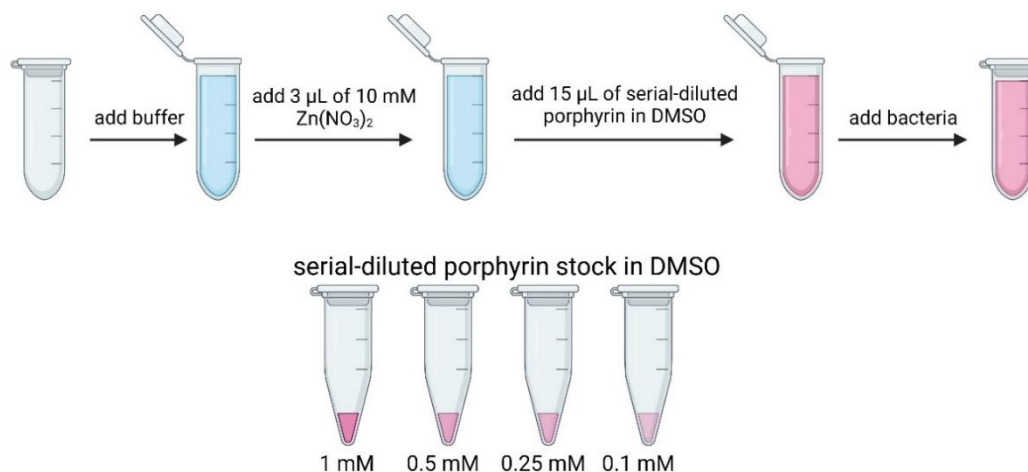


Figure 12. Diagram of preparation of bacterial suspensions for irradiation and dark treatment in first generation approach

Light control samples were prepared with the same composition as the experimental samples, but without the addition of the photosensitizer. Dark control samples were prepared with the photosensitizer in the same concentration range as the experimental samples, but they were not exposed to light. The bacterial suspensions were incubated in the dark at room temperature for 20 minutes to allow for pre-incubation before irradiation.

By following these steps, the bacterial suspensions with the desired concentration and the appropriate photosensitizer concentration were prepared for subsequent photoinactivation experiments. The light control and dark control samples were also prepared for comparison purposes.

2.2.4 Irradiation of Bacterial Suspensions in the Presence of Oxygen

In bacterial photoinactivation studies, a 400 W halogen bulb was used as the light source. The light emitted from the bulb was filtered to ensure specific characteristics.

Firstly, a cooling filter made of plexiglass was used, and cold water was circulated through it to prevent overheating. Secondly, a green filter was applied to remove all emissions below 400 nm. This filtration process helped to achieve the desired wavelength range for effective bacterial photoinactivation. During the experiments, the bacterial suspensions were positioned at a distance of 25 cm from the light source. This distance ensured that the desired fluence of 34 mW/cm² was delivered to the bacterial samples. The experiments were conducted at room temperature, meaning the bacterial suspensions were maintained at the ambient temperature of the laboratory during the photoinactivation process.

To conduct the bacterial inactivation studies, the following steps were performed:

In each well of a sterile 96-well culture plate, 250 µL aliquots of the bacterial suspensions prepared in the previous section were added. This step was carried out in three replicates. The bacterial suspensions in the wells were irradiated from the top for a duration of 60 minutes at room temperature. For each bacterial strain and concentration of **1d**, at least three independent experiments were performed to obtain reliable data. Dark control suspensions, which were not exposed to light, were kept in a dark place for 60 minutes.

After the irradiation period, the irradiated bacterial suspensions, dark controls, and light controls were appropriately diluted to obtain samples with theoretical bacterial cell numbers of 10⁶, 10⁵, 10⁴, and 10³ CFU/mL. From each diluted sample, 50 µL was plated on 90 mm petri dishes containing sterile LB agar. The petri dishes were then incubated at 37 °C in the dark for approximately 18 hours. After incubation, the colonies formed on the agar plates were counted to determine the photoinactivation efficacy and dark toxicity data.

By following these steps, the efficacy of photoinactivation and the potential dark toxicity of the treatment was evaluated by counting the colonies formed on the agar plates. The experiments were conducted in replicates, and appropriate controls were included to compare the results accurately. At least three independent experiments were performed to obtain reliable data for each bacterial strain and concentration of

1d. Colony count on the dark DMSO controls was used as calibration control and results were applied student t-test at 95% confidence level. ($p \leq 0.05$)

2.2.5 Zinc Toxicity on Bacterial Cells

For *Escherichia coli* Top10, $\text{Zn}(\text{NO}_3)_2$ toxicity experiments were done as follows:

In a sterile 2 mL Eppendorf tube, a 10^8 CFU/mL bacterial suspension was prepared by taking the proper OD_{600} relationship into account. 75 μL from the suspension was added into another sterile Eppendorf tube containing 1422 μL of 10 mM HEPES, 15 mM NaCl pH 7.4 buffer. Into this tube, 3 μL $\text{Zn}(\text{NO}_3)_2$ stock in DI water was added. $\text{Zn}(\text{NO}_3)_2$ stocks had concentrations in the 10 mM to 1 mM range to give a final concentration range of 2-20 μM in the experimental samples. After keeping the Eppendorf tubes in the dark for a total of 80 minutes, the bacterial suspension was diluted to 10^3 CFU/mL. From each diluted sample, 50 μL was plated on 90 mm petri dishes containing sterile LB agar. The plates were incubated at 37 °C in the dark for approximately 18 hours. After incubation, the colonies formed on the agar plates were counted to determine the toxicity of $\text{Zn}(\text{NO}_3)_2$.

For *Staphylococcus aureus* ATCC 25923 the $\text{Zn}(\text{NO}_3)_2$ toxicity experiments were done as follows:

In a sterile 2 mL Eppendorf tube, a 5×10^7 CFU/mL bacterial suspension was prepared by taking the proper OD_{600} relationship into account. 30 μL from the suspension was added into another sterile Eppendorf tube containing 1467 μL of 10 mM HEPES, 15 mM NaCl pH 7.4 buffer. Into this tube, 3 μL $\text{Zn}(\text{NO}_3)_2$ stock in DI water was added. $\text{Zn}(\text{NO}_3)_2$ stocks had a concentration range from 2 mM to 0.25 mM to give a final concentration range of 4-0.5 μM in the experimental samples. After keeping the Eppendorf tubes in the dark for a total of 120 minutes, the bacterial suspensions were diluted to 10^4 CFU/mL. From each diluted sample, 25 μL was plated on 80 mm petri dishes containing sterile LB agar. The plates were incubated at 37 °C in the dark for

approximately 18 hours. After incubation, the colonies formed on the agar plates were counted to determine the toxicity of $\text{Zn}(\text{NO}_3)_2$.

2.2.6 Second Generation of Preparation of Bacterial Suspensions for Irradiation and for Dark Treatment

To prepare the bacterial suspensions for photoinactivation experiments, the following steps were followed:

In a sterile 2 mL Eppendorf tube, an appropriate volume from 15 mM NaCl, 10 mM HEPES pH 7.4 buffer was added so that the final volume would be 1500 μL at the end. For every desired final concentration of porphyrin, serial dilution was performed to obtain a stock solution with 100 times greater concentration than the desired final experimental concentration. 15 μL of thus prepared stock solutions of Zn_2BDPA -porphyrin derivative **1d** in DMSO was added into a 2 mL sterile Eppendorf tube. Then, 30 μL of a bacterial suspension with a bacterial cell number of 5×10^7 CFU/mL was added to the tube. Thus, the final bacterial suspensions had a concentration of 1×10^6 CFU/mL. The final concentration of the photosensitizer in the suspensions was in the 1-20 μM range (Figure 13).

Light control samples were prepared with the same composition as the experimental samples, but without the addition of the photosensitizer. Dark control samples were prepared with the photosensitizer in the same concentration range as the experimental samples, but they were not exposed to light. The bacterial suspensions were incubated in the dark at room temperature for 20 minutes to allow for pre-incubation before irradiation.

By following these steps, the bacterial suspensions with the desired concentration and the appropriate photosensitizer concentration were prepared for subsequent photoinactivation experiments. The light control and dark control samples were also prepared for comparison purposes. At least three independent experiments were performed to obtain reliable data for each bacterial strain and concentration of **1d**.

After the irradiation period, the bacterial suspensions, dark controls, and light controls were diluted to obtain samples with theoretical bacterial cell numbers ranging from 10^6 to 10^3 CFU/mL. These diluted samples were then plated onto sterile LB-agar petri dishes in 25 μ L aliquots. The petri dishes were incubated in the dark at 37 °C for approximately 18 hours. Following the incubation, the colonies formed on the agar plates were counted to evaluate the effectiveness of photoinactivation and the potential dark toxicity of the treatment.

By employing these steps, the photoinactivation efficacy and dark toxicity of the treatment were evaluated through colony counting on agar plates. The experiments were performed in replicates, and appropriate controls were included to ensure accurate comparison of the results. A minimum of three independent experiments were conducted for each bacterial strain and concentration of **1d** to ensure reliable data. The colony count on the dark DMSO controls served as the calibration control, and the results were subjected to a student t-test at a 95% confidence level ($p \leq 0.05$).

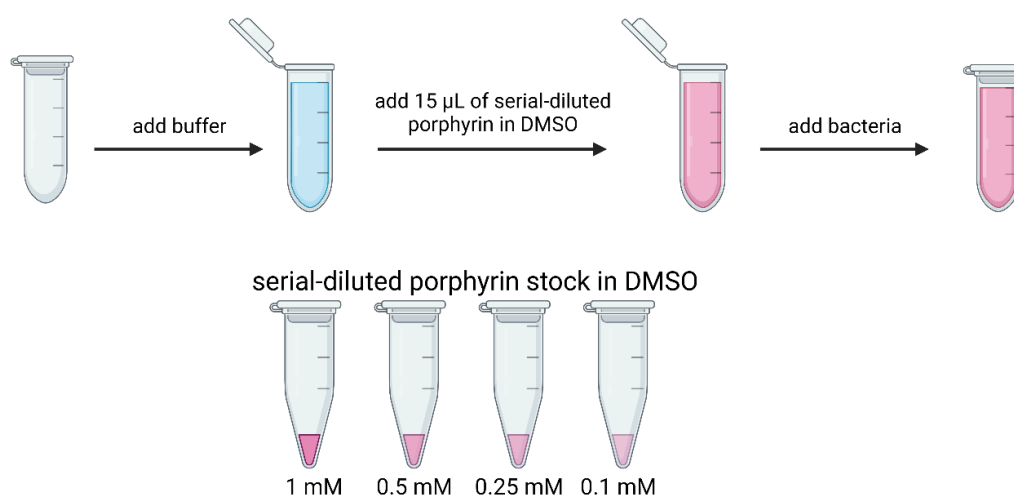


Figure 13. Diagram of preparation of bacterial suspensions for irradiation and dark treatment in second-generation approach.

2.3 Broth Microdilution Experiments

2.3.1 Broth Microdilution Procedure for Antibiotic Assays

An antibiotic stock that was 100 times greater than the desired concentration was prepared, following the guidelines outlined in the CLSI standards.¹¹⁶ The stock was divided into Eppendorf tubes and stored at a temperature below -60 °C. The stock was thawed only once, and any remaining thawed solution was discarded. Cells were harvested by centrifuging 2 mL of the overnight-grown inoculum in a sterile 2 mL aliquot of LB. The supernatant was removed, and this procedure was repeated once more. The bacterial pellet was resuspended by adding 1 mL of 10 mM HEPES pH 7.4 buffer containing 15 mM NaCl and resuspending it through pipetting. A waiting period of 2-4 minutes was allowed, followed by centrifugation and removal of the supernatant. This washing step was repeated three additional times. An aliquot of 100 µL of the bacterial suspension was diluted to a final volume of 2 mL inside the sample cell for UV-Vis measurement. The UV-Vis absorbance at 600 nm was measured. The CFU/mL concentration was adjusted according to the OD₆₀₀ of the bacterial strain to achieve the desired bacterial cell number. A concentration of 10⁶ CFU/mL was prepared in Cation-adjusted Mueller-Hinton Broth. A sterile 96-well plate was taken, and 50 µL of antibiotic that was four times larger concentration than the desired concentration was added into the first cell. In all the cells of the row, including the one containing the antibiotic, 50 µL of Cation-Adjusted Mueller-Hinton Broth (CAMHB) was added. From cell 1, 50 µL was taken and diluted by adding it to cell 2, which already contained 50 µL of broth. Then, 50 µL was transferred from this cell to the next cell, and this serial dilution process was continued until the last cell in the row. Finally, 50 µL from the last cell was discarded, ensuring that each cell in the row contained 50 µL. Consequently, the resulting concentration was twice the desired concentration at this step. 50 µL from the 10⁶ CFU/mL bacteria in CAMHB was taken and added to all the prepared cells. This achieved the desired concentration of all agents in each cell. An empty cell was

filled with 100 μL of Cation-Adjusted Mueller-Hinton Broth to serve as the sterility control. In another cell, a combination of 50 μL of the 10^6 CFU/mL bacteria in broth with 50 μL of broth was prepared to establish the growth control. The 96-well plate was incubated at a temperature of 35 $^{\circ}\text{C}$ for 18-20 hours. The UV-Vis absorbance at 630 nm was measured using a plate reader for MIC concentrations. For MBC, streaking was performed from cells with 0.000 absorbances by taking 10 μL from solutions and plating them on the LB-Agar plate. Experiments for each concentration of each molecule were repeated as independent experiments at least three times.

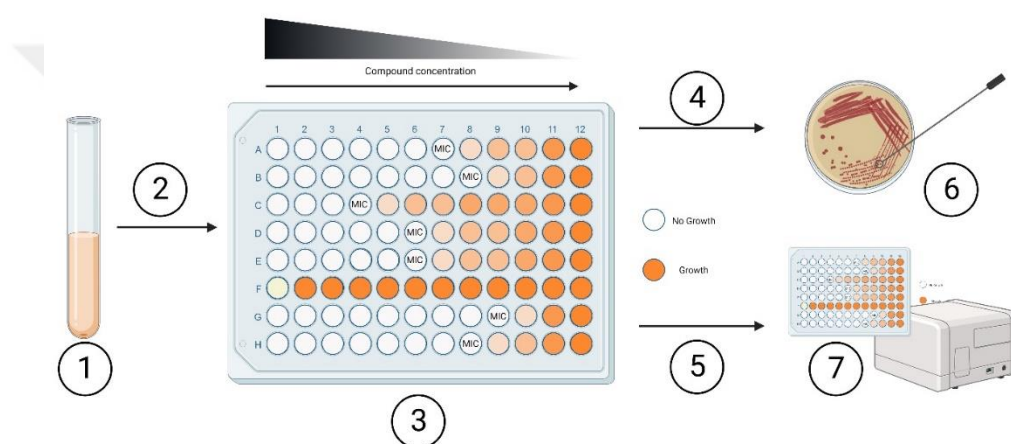
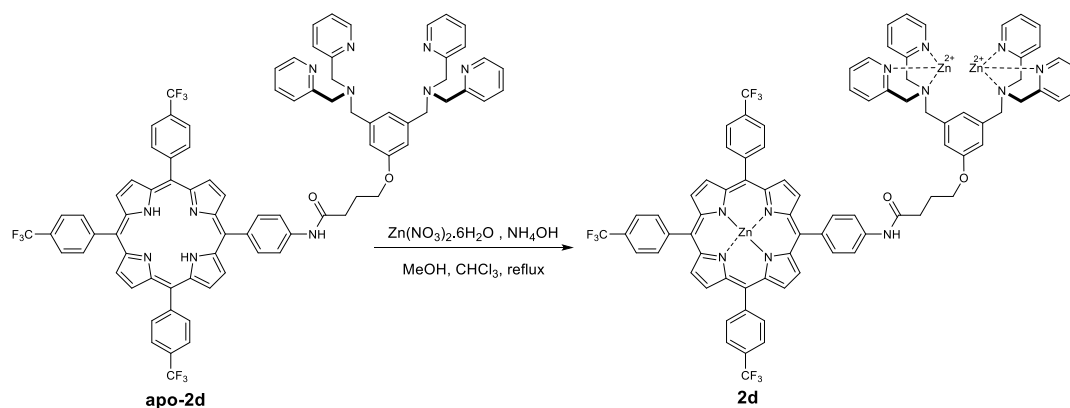


Figure 14. Schematic diagram of broth microdilution procedure.

2.3.2 Broth Microdilution for Photodynamic Inactivation on Bacteria

Bacterial strains used in the following experiments are *Staphylococcus aureus* ATCC 25923, *Bacillus cereus* ATCC 14579, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 9027.

2.3.3 Metalation of PDT Agent apo-2



To a solution of 5 mg (3.5 μmol) of **apo-2**, synthesized by another student¹¹⁵, in 2 mL chloroform (CHCl_3) was added 3.12 mg (10 μmol) of $(\text{Zn}(\text{NO}_3)_2$ in 200 μL methanol and 7.05 μmol of NH_4OH in 20 μL methanol. The reaction was refluxed for 2 hours, and a slight color change was observed from purple to lighter purple. After the reaction was complete, the solvent was removed *in vacuo*. UV-Vis spectrum was taken in order to investigate if the reaction is complete and full metalation of the porphyrin core is observed.

2.3.4 Broth Microdilution for Testing of Photodynamic Inactivation of Bacteria

Bacterial suspensions containing 10^6 CFU/mL bacteria and the desired photosensitizer concentration of **2** were prepared as mentioned before.

A volume of 200 μL was taken from this suspension and placed into a 96-well plate in 3 aliquots. Dark controls were placed in the dark. The 96-well plate was irradiated for 90 minutes. After irradiation, 100 μL of the light controls were discarded. A total of 100 μL from the dark controls was placed into a 96-well plate in 3 aliquots. Every cell containing dark and light controls was supplemented with 100 μL of Cation-Adjusted Mueller-Hinton Broth. The 96-well plate was incubated at a temperature

of 35 °C for 18-20 hours. After incubation, 96-well plate cells that did not show turbidity were plated on LB-Agar to determine the MBC value, and UV-Vis measurements at 630 nm were taken using a plate reader to determine the presence or absence of bacterial growth. Experiments for each concentration of **2** were repeated as independent experiments at least three times.

2.4 Liposome Preparation

Liposomes are prepared by the lipid film hydration/extrusion method. A thin film was first prepared by mixing appropriate amounts of lipophilic agents as CHCl_3 solutions and removal of the organic solvents under a stream of nitrogen gas. These films were hydrated with 1 mL of 10 mM HEPES (137 mM NaCl, 3.2 mM KCl, 1 μM $\text{Zn}(\text{NO}_3)_2$ pH 7.4) buffer and agitating using a vortex mixer. The hydrated lipid films were subjected to 6 freeze-thaw cycles ($-195\text{ }^\circ\text{C} \leftrightarrow 40\text{ }^\circ\text{C}$) followed by extrusion through 200 nm track-etched polycarbonate filters.

2.4.1 Thin Film Formation

For thin film formation, cholesterol, $\text{Zn}_2\text{BDPA-CF}_3\text{-Porphyrin}$ (**2**), POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine), and rifampicin stocks were prepared as follows:

- 63.6 mg cholesterol dissolved in 10 mL CHCl_3 to give a 16.45 mM (6.36 mg/mL) solution.
- 125 mg POPC was dissolved in 10 mL CHCl_3 to give a 16.45 mM (12.5 mg/mL) solution.
- 6.5 mg (3.38×10^{-6} mol) of $\text{Zn}_2\text{BDPA-CF}_3\text{-Porphyrin}$ (**2**) in 20 mL $\text{CHCl}_3\text{:MeOH}$ (3:1) to give a 0.169 mM (0.325 mg/mL) solution.
- 50 mg (6.08×10^{-5} mol) of rifampicin was dissolved in 10 mL chloroform to give 6.08 mM (5 mg/mL) solution.

2.4.2 Lipid Film Hydration and Liposome Formation

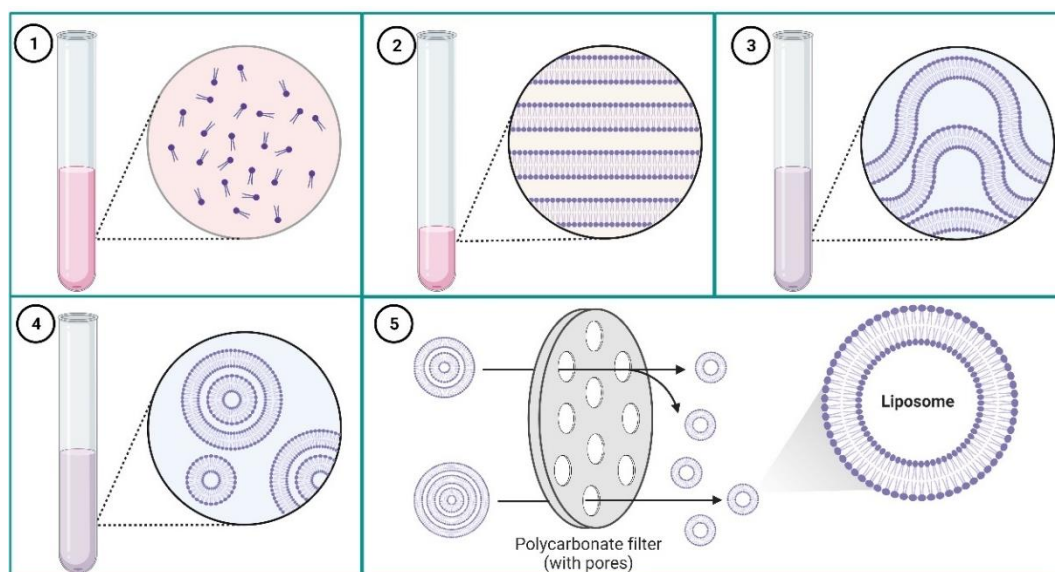


Figure 15. Schematic diagram of liposome preparation by thin film hydration and extrusion.

The objective was to prepare stock liposome dispersions with a total lipid concentration of 3.32 mM in 1 mL volume. To achieve this, lipid solutions with compositions specified in Table 1 were prepared in 13x100 mm test tubes (Step 1 in Figure 15). The solvent was then removed by gently blowing nitrogen over the solutions, resulting in the formation of lipid films (Step 2). These films were dried under vacuum overnight. The dried lipid films were subsequently hydrated with 1000 μ l of HEPES pH 7.4 buffer (Step 3). To ensure proper MLV (i.e., multilamellar vesicle) formation, the hydrated films underwent 6 cycles of freeze-thawing (i.e., alternating between 40 °C and liquid nitrogen). Following this, the resulting multilamellar vesicles (i.e., MLVs) were extruded by passing them through a 200 nm Nucleopore filter 21 times, resulting in the formation of liposomes (i.e., SUVs, small unilamellar vesicles) (Steps 4 and 5). Characterization was done by DLS and Zeta potential measurements.

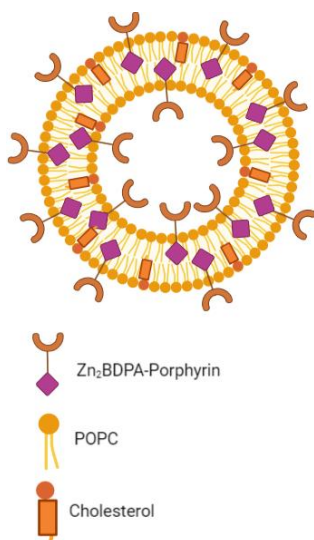


Figure 16. Schematic diagram of liposomes.

Table 1. Lipid composition for liposomes containing 2 mol% $\text{Zn}_2\text{BDPA-CF}_3\text{-Porphyrin (2)}$, 3.32 μmol total lipid.

	[Lipid] _{stock} (mM)	Volume (μl)	Moles (μmol)	Mol %
$\text{Zn}_2\text{BDPA-CF}_3\text{-Porphyrin (2)}$	0.31	212.9	0.066	2
POPC	16.45	137.3	2.258	68
Cholesterol	16.45	60.8	1.00	30

Table 2. Lipid composition for liposomes containing 2 mol% $\text{Zn}_2\text{BDPA-CF}_3\text{-Porphyrin (2)}$, 3.32 μmol total lipid. No cholesterol was present in this formulation.

	[Lipid] _{stock} (mM)	Volume (μl)	Moles (μmol)	Mol %
$\text{Zn}_2\text{BDPA-CF}_3\text{-Porphyrin (2)}$	0.17	388.2	0.066	2
POPC	16.45	198.1	3.258	98

Table 3. Lipid composition for liposomes containing 5 mol% Zn₂BDPA-CF₃-Porphyrin (2), 3.32 μ mol total lipid. No cholesterol. This formulation gives [Zn₂BDPA-CF₃-Porphyrin] = 0.166 mM in 1 mL liposomal dispersion.

	[Lipid] _{stock} (mM)	Volume (μ l)	Moles (μ mol)	Mol %
Zn₂BDPA-CF₃-Porphyrin (2)	0.17	970.5	0.166	5
POPC	16.45	191.7	3.154	95

Table 4. Lipid composition for liposomes containing 10 mol% Rifampicin and 5 mol% Zn₂BDPA-Chol, 10 μ mol total lipid. No cholesterol. This formulation gives [Rifampicin] = 1 mM in 1 mL liposomal dispersion.

	[Lipid] _{stock} (mM)	Volume (μ l)	Moles (μ mol)	Mol %
Rifampicin	6.08	164.5	1	10
Zn₂BDPA-Chol	1.15	435.8	0.5	5
POPC	16.45	516.7	8.5	85

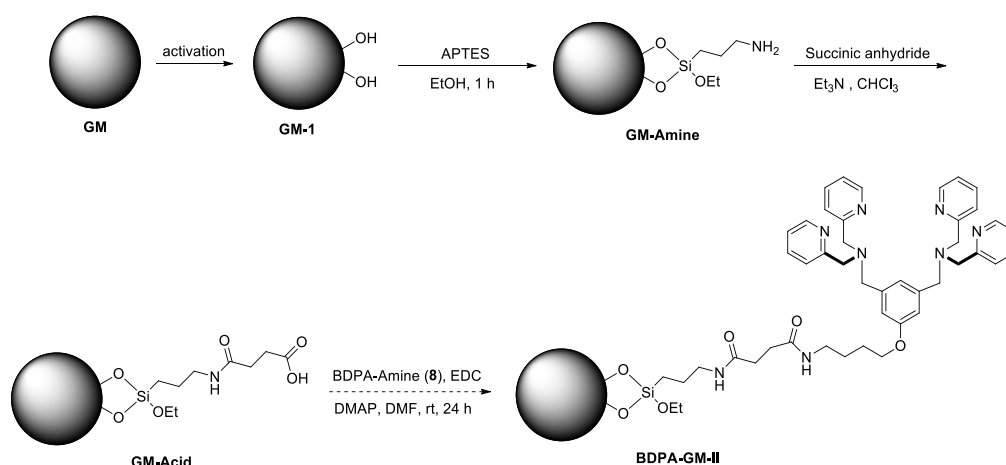
2.5 Glass Derivatizations

2.5.1 Glass Microspheres

Glass microspheres were purchased from Swarco as “Swarcoblast Glass Blasting Beads” 150-250 μ m, Soda-Lime Glass. Their chemical composition was SiO₂ 68.0–75.0%, Al₂O₃ 0–2.5%, MgO 0–5.0%, CaO 7.0–12.0%, Na₂O 12.0–18.0%, other max. 2.0%. Density of glass microspheres was 2.5 g/cm³.

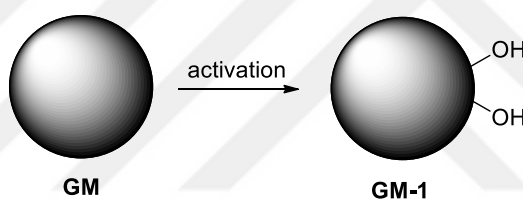
2.5.2 Strategy I for Glass Derivatization

The synthetic approach towards BDPA-GM-I is described in Scheme 1.



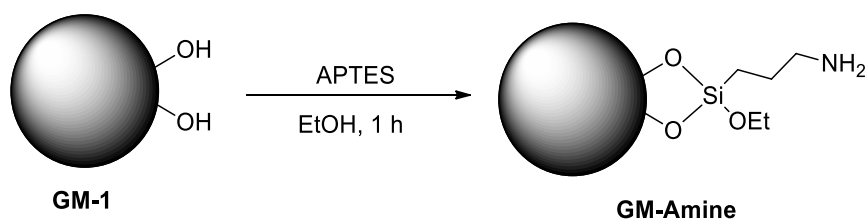
Scheme 1: Proposed preparation of BDPA-GM-I.

2.5.2.1 Activation of Glass Microspheres



20 mL of Piranha solution was prepared by adding slowly 5 mL of 30% H₂O₂ to 15 mL of concentrated H₂SO₄ in a beaker. 45 g of 150-250 μM glass microspheres were added and the mixture was heated up to 80 °C. After 90 minutes, mixture was cooled down and diluted to 500 mL with deionized water and neutralized with NaOH pellets. Glass microspheres were filtered and washed with deionized water (i.e., DI H₂O) and ethanol and were dried at 60 °C.

2.5.2.2 Silanization Reaction of Activated Glass Microspheres by APTES



6.0 g of activated glass microspheres were added in a vial containing 15 mL of ethanol. 300 mg (3-aminopropyl)triethoxysilane (APTES) was added to obtain 5% (APTES/microspheres, w/w). Reaction was stirred at 80 °C for 1 hour. Glass microspheres were filtrated and washed with ethanol and were dried at 60 °C. For the characterization of GM-Amine spheres, Kaiser test was performed.

2.5.2.3 Kaiser Test

Ninhydrin test is performed to detect primary amine groups and the test was positive, as a purple color was observed. Thus, the Kaiser Test was performed to calculate primary amine groups. Reagent preparation and the test were executed as follows:

Reagent A: To a 49 mL of pyridine was added 1 mL of 1.65 mg of KCN in 25 mL of deionized water.

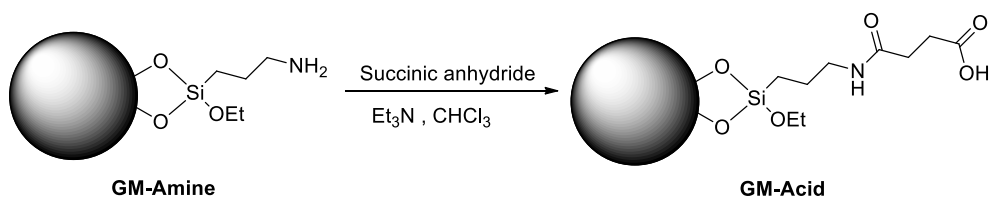
Reagent B: 20 g phenol in 10 mL of EtOH.

Reagent C: 0.5 g ninhydrin in 10 mL EtOH.

To 200 mg spheres, 1 mL of DI water, 200 μL of Reagent A, and 150 μL of Reagent B were added and stirred with a vortex stirrer. Then, 150 μL of Reagent C and 500 mL ethanol was added. The reaction was heated with a 97 °C water bath. The supernatant was taken and its UV-Vis spectrum was recorded.

For the calibration curve, a final concentration of 0.01, 0.02, 0.03, 0.05, 0.06, 0.07, 0.1, 0.25, 0.5, and 0.1 mM stocks of n-hexylamine with the procedure above was prepared and UV-Vis measured.

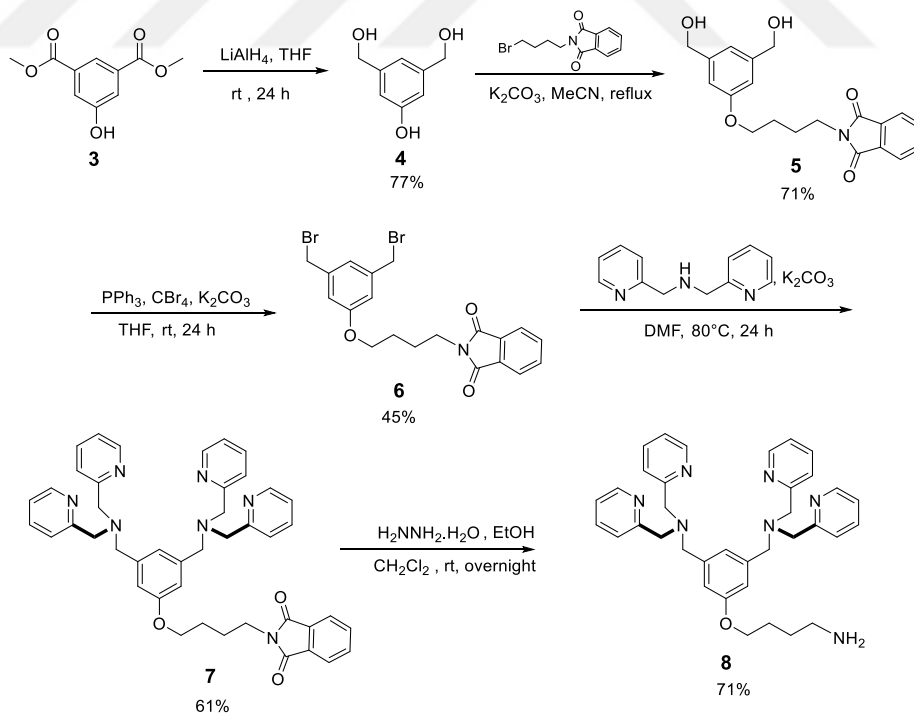
2.5.2.4 Succinic Anhydride Reaction



To 0.6 g of GM-Amine spheres in 20 mL chloroform was added 0.8 g succinic anhydride and 1.2 mL of triethyl amine. The reaction was continued for 24 hours. Glass microspheres were filtered and washed with ethanol. After overnight drying at 60 °C, an ATR-FTIR spectrum of GM-acid was taken for characterization.

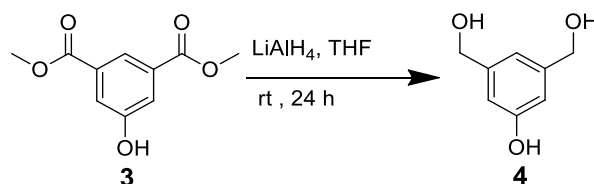
2.5.2.5 Synthesis of BDPA-Amine Ligand (8)

Preparation of BDPA-GM-1 required coupling of GM-Acid and BDPA-Amine (8). BDPA-Amine (8) was synthesized as summarized in Scheme 2.



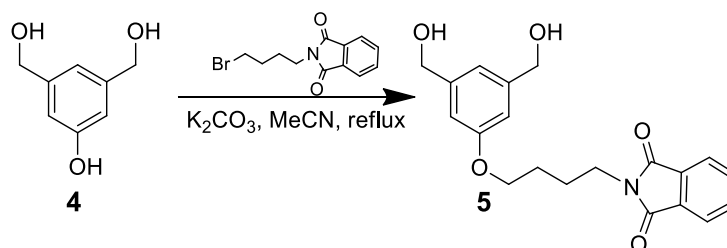
Scheme 2: Preparation of BDPA-Amine (8).

2.5.2.5.1 Reduction of 5-Hydroxyisophthalate (3)



1.08 g (28.5 mmol) of LiAlH_4 was added to a round-bottom flask that contained 40 mL anhydrous THF under N_2 atmosphere. Into the dispersion was added 3.00 g (14.2 mmol) of 5-hydroxyisophthalate (3) under N_2 atmosphere dropwise. The reaction was stirred for 24 h at room temperature. Once the reaction was completed, it was cooled to a temperature of 0°C using an ice bath. Subsequently, the reaction was quenched by dropwise adding 20 mL of 10% H_2SO_4 . The solvent was removed *in vacuo*. The residue was extracted by using 100 mL saturated brine and 100 mL EtOAc. The aqueous phase was washed with 50 mL of EtOAc 5 more times. Then, the combined organic phase was dried with MgSO_4 , and the solvent was removed *in vacuo*. 1.70 g (11.0 mmol, 77% yield) of 4 was obtained as a white crystal. R_f : 0.51 (SiO_2 , EtOAc).

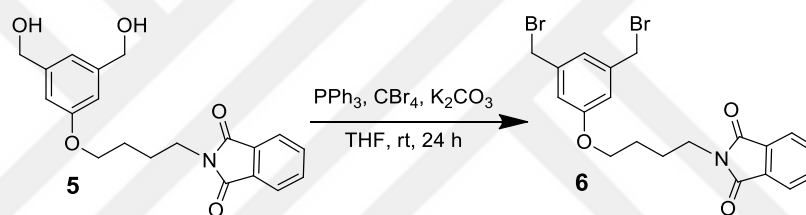
2.5.2.5.2 2-(4-(3,5-Bis(hydroxymethyl)phenoxy)butyl)isoindoline-1,3-dione (5)



A solution of 1.43 g (5.06 mmol) of N-(4-bromobutyl)phthalimide in 5 mL of acetonitrile (MeCN) was added dropwise to a mixture of 650 mg (4.22 mmol) of compound 4 and 3.90 g (28.2 mmol) of potassium carbonate (K_2CO_3) in 15 mL MeCN. The reaction mixture was refluxed overnight using a reflux condenser

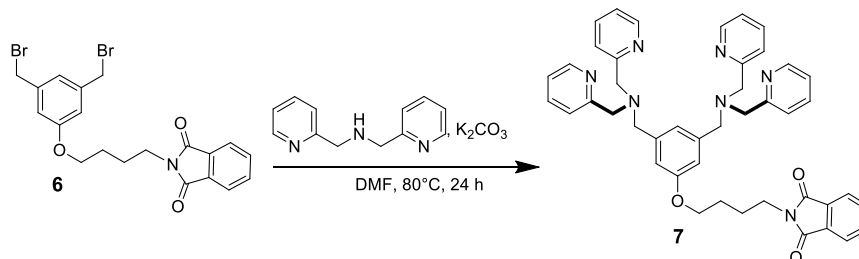
equipped with a drying tube containing calcium chloride (CaCl_2). After the reaction was completed, the solvent was removed *in vacuo*. The resulting residue was then extracted with 20 mL of chloroform (CHCl_3) and 20 ml of water (H_2O). The aqueous phase was extracted twice more with 20 mL of CHCl_3 , and the combined organic phase was dried with Na_2SO_4 . the solvent was removed *in vacuo*. The residue was chromatographed on SiO_2 with EtOAc. 1.06 g (3 mmol, 71% yield) pure product was obtained in a white crystal solid. R_f : 0.45 (SiO_2 , EtOAc). ^1H NMR (400 MHz, CDCl_3) δ ppm 1.77 - 1.96 (m, 4H) 3.77 (t, $J=6.78$ Hz, 2 H) 4.03 (t, $J=5.90$ Hz, 2H) 4.66 (s, 4H) 6.84 (s, 2H) 6.93 (s, 1H) 7.67 - 7.79 (m, 2H) 7.80 - 7.91 (m, 2H).

2.5.2.5.3 2-(4-(3,5-Bis(bromomethyl)phenoxy)butyl)isoindoline-1,3-dione (6)



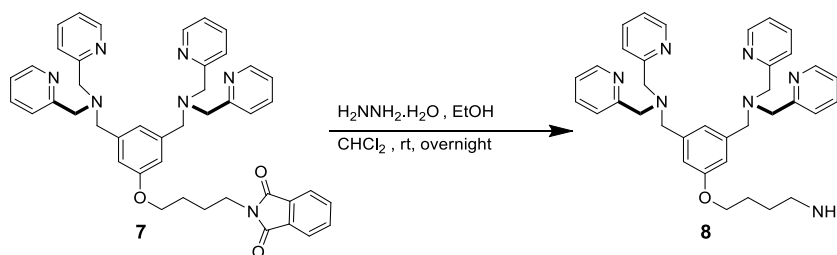
A solution of 1.73 g (5.21 mmol) of carbon tetrabromide, CBr_4 , in 4 mL anhydrous THF was added dropwise to a mixture of 738 mg (2.09 mmol) of **5**, 1.37 g (5.21 mmol) of triphenylphosphine (PPh_3), and 1.44 g (10.4 mmol) of potassium carbonate (K_2CO_3) in 4 mL anhydrous THF under N_2 atmosphere. The reaction was carried out for 24 hours at room temperature under N_2 atmosphere. After the reaction was completed, the reaction was filtered to get rid of potassium carbonate. The solvent was removed *in vacuo*. The material was purified by flash column chromatography (SiO_2 , 1:1 EtOAc:Hexane). 449 mg of pure **6** was obtained. 0.94 mmol, 45% yield). R_f : 0.47 (SiO_2 , 1:1 EtOAc:Hexane). ^1H NMR (400 MHz, CDCl_3) δ ppm 1.81 - 1.97 (m, 4H) 3.78 (t, $J=6.78$ Hz, 2H) 4.01 (t, $J=5.90$ Hz, 2H) 4.42 (s, 4H) 6.84 (s, 2H) 6.99 (s, 1H) 7.69 - 7.79 (m, 2H) 7.83 - 7.91 (m, 2H).

2.5.2.5.4 2-(4-(3,5-Bis((bis(pyridin-2-ylmethyl)amino)methyl)phenoxy)-butyl)isoindoline-1,3-dione (7)



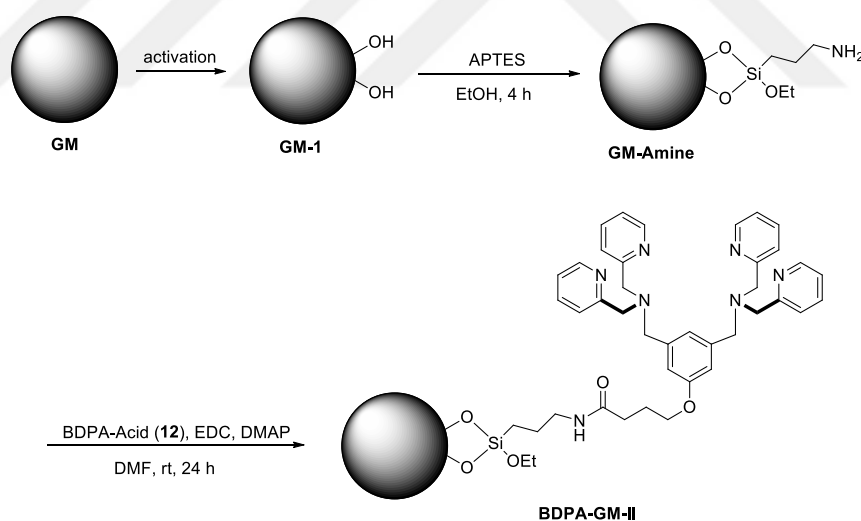
A 365 μL of dipicolylamine (405 mg, 2.03 mmol) was added into the mixture of 443 mg (0.92 mmol) of **6**, and 1.28 g (9.25 mmol) of potassium carbonate (K_2CO_3) in 20 mL anhydrous DMF under N_2 atmosphere. The reaction was carried out under N_2 atmosphere at 80 $^\circ\text{C}$ for 24 hours. After the reaction was complete, the solvent was removed *in vacuo*. The residue was partitioned between 25 mL of saturated brine and 25 mL of CHCl_3 . The aqueous phase was washed with 25 mL of CHCl_3 twice more. The combined organic phase was dried with Na_2SO_4 . And solvent was removed *in vacuo*. The residue was subjected to flash column chromatography (SiO_2 , 8:1:0.1 CHCl_3 :MeOH: NH_4OH). 405 mg (0.56 mmol, 61% yield) of **7** was obtained. R_f : 0.44 (SiO_2 , 8:1:0.1 CHCl_3 :MeOH: NH_4OH). ^1H NMR (400 MHz, CDCl_3) δ ppm 1.78 - 1.96 (m, 4H) 3.63 (s, 4H) 3.73 - 3.85 (m, 11H) 3.92 - 4.02 (m, 2H) 6.85 (s, 2H) 7.05 (s, 1H) 7.10-7.20 (m, 4H) 7.58 - 7.68 (m, 8H) 7.69 - 7.76 (m, 2H) 7.80 - 7.88 (m, 2H) 8.51 (d, $J=4.02$ Hz, 4H).

2.5.2.5.5 N,N'-((5-(4-Aminobutoxy)-1,3-phenylene)bis(methylene))bis(1-(pyridin-2-yl)-N-(pyridin-2-ylmethyl)methanamine) (8)



A solution of 167 mg (0.23 mmol) of compound **7** in 4.3 ml of ethanol (EtOH) and 1.1 ml of dichloromethane (DCM) was prepared. To this solution, 340 μ l (0.34 mg, 6.70 mmol) of hydrazine monohydrate was added dropwise under N₂ atmosphere. The reaction mixture was then magnetically stirred overnight at room temperature under N₂ atmosphere. After completion of the reaction, the reaction mixture was diluted with 10 mL of DCM, filtered, and the solvent was evaporated *in vacuo*. The resulting residue was purified using a gradient FCC (SiO₂, CHCl₃ to 8:2:0.2 CHCl₃:MeOH:NH₄OH). 96 mg of **8** was obtained (71% yield). R_f: 0.49 (SiO₂, 8:2:0.2 CHCl₃:MeOH:NH₄OH). ¹H NMR (400 MHz, CDCl₃) δ ppm 1.84 - 1.94 (m, 2H) 2.04 (d, *J*=7.78 Hz, 3H) 3.15 (t, *J*=7.28 Hz, 2H) 3.57 - 3.67 (m, 4H) 3.72 (d, *J*=4.77 Hz, 1H) 3.81 (br s, 7H) 3.95 (t, *J*=5.65 Hz, 2H) 6.77 (br s, 2 H) 6.91 (s, 1H) 7.09 - 7.19 (m, 4H) 7.45 - 7.71 (m, 8H) 8.44 - 8.60 (m, 4H).

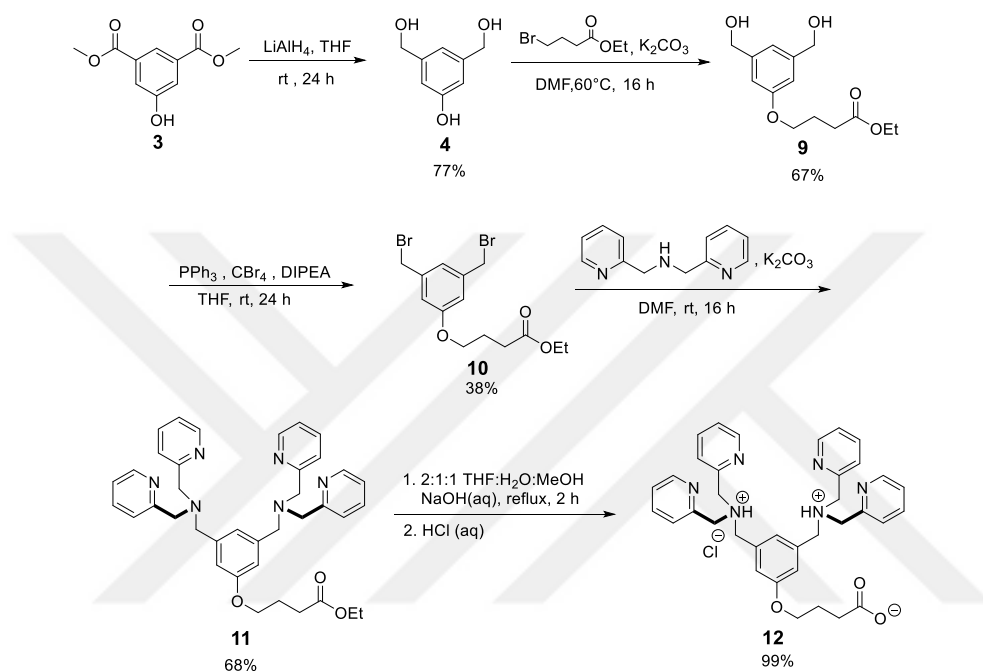
2.5.3 Strategy II for Glass Microsphere Derivatization



Scheme 3: Proposed preparation of BDPA-GM-II.

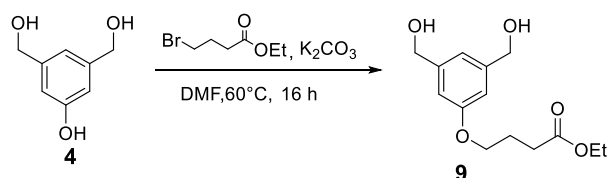
2.5.3.1 BDPA-Acid (12) Ligand Preparation

Preparation of BDPA-GM-II required BDPA-acid (12). This material was synthesized according to Scheme 4.



Scheme 4: Preparation of BDPA-Acid (12).

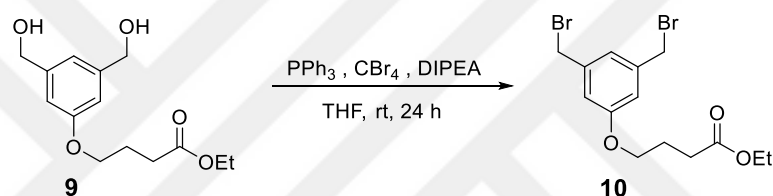
2.5.3.1.1 Ethyl 4-(3,5-bis(hydroxymethyl)phenoxy)butanoate (9)



A solution containing 500 mg (3.24 mmol) of (5-hydroxy-1,3-phenylene)dimethanol (4) and 1.89 g (9.73 mmol) of ethyl 4-bromobutyrate in 10 mL of anhydrous DMF was prepared. To this solution, 2.24 g (16.2 mmol) of K_2CO_3 was added and the mixture was stirred at 60°C under N_2 atmosphere for 16 hours. The solvent was then

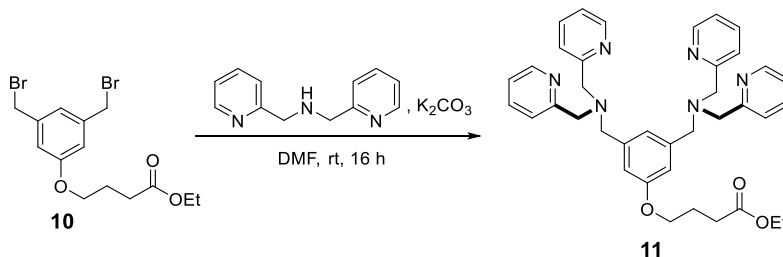
evaporated *in vacuo*, and the residue was partitioned between 25 mL of DI water and 25 mL of EtOAc. The aqueous phase was washed twice with 25 mL of EtOAc each. The combined organic phase was dried using MgSO₄. After removing the solvent, the remaining mixture was subjected to flash column chromatography (SiO₂, EtOAc), resulting in the isolation of 580 mg (67% yield) of the desired compound as pale yellow oil. R_f: 0.49 (SiO₂, EtOAc). ¹H NMR (400 MHz, CDCl₃) δ ppm 1.27 (t, *J*=7.15 Hz, 3H) 2.11 (quin, *J*=6.71 Hz, 2H) 2.52 (t, *J*=7.28 Hz, 2H) 4.03 (t, *J*=6.02 Hz, 2H) 4.15 (q, *J*=7.28 Hz, 2H) 4.67 (s, 4H) 6.84 (s, 2H) 6.94 (s, 1H).

2.5.3.1.2 Ethyl 4-(3,5-bis(bromomethyl)phenoxy)butanoate (10)



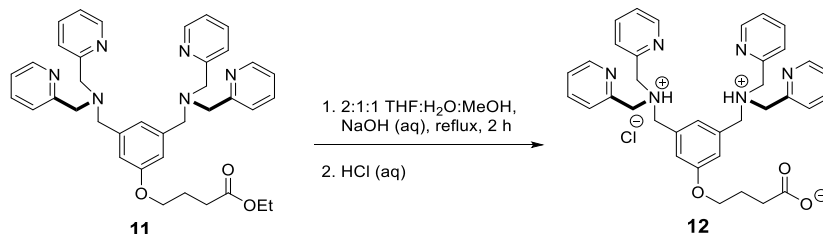
A solution containing 500 mg (1.86 mmol) of ethyl 4-(3,5-bis(hydroxymethyl)phenoxy)butanoate, 1.22 g (4.66 mmol) of PPh₃, and 600 mg (4.66 mmol) of DIPEA in 2 mL of anhydrous THF was prepared under N₂ atmosphere. To this solution, 1.54 g (4.66 mmol) of CBr₄ dissolved in 5 mL of anhydrous THF was added dropwise. The reaction mixture was then allowed to react at room temperature under an N₂ atmosphere for 16 hours. The reaction was filtrated, and the solvent was evaporated *in vacuo*. The residue was washed three times with 5 mL of EtOAc. The solvent was evaporated *in vacuo*, the residue was subjected to flash column chromatography (SiO₂, 1:1 EtOAc:Hexane). This purification process yielded 278 mg (38% yield) of **10** as pale-yellow oil. R_f: 0.72 (SiO₂, 1:1 EtOAc:Hexane). ¹H NMR (400 MHz, CDCl₃) δ ppm 1.26 - 1.31 (m, 3H) 2.14 (quin, *J*=6.65 Hz, 2H) 2.54 (t, *J*=7.28 Hz, 2H) 4.04 (t, *J*=6.02 Hz, 2H) 4.18 (q, *J*=7.03 Hz, 2H) 4.43 - 4.48 (m, 4H) 6.87 (s, 2H) 7.02 (s, 1H).

2.5.3.1.3 Ethyl 4-(3,5-Bis(pyridine-2-ylmethyl)amino)methylphenoxy)-butanoate (**11**)



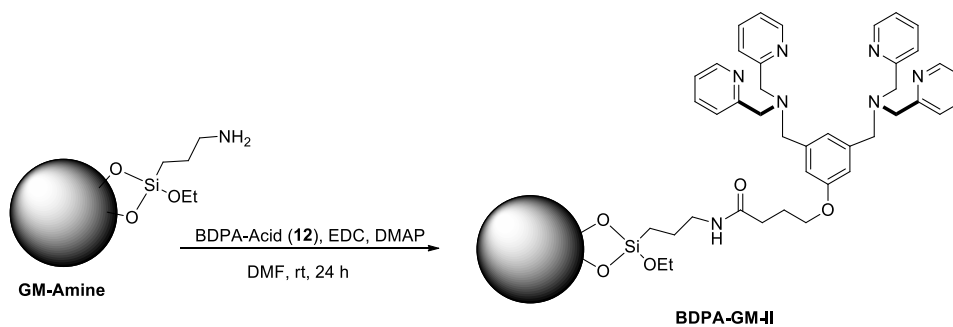
A mixture of 110 mg (0.28 mmol) of compound **10** and 192 mg (0.61 mmol) of potassium carbonate (K_2CO_3) in 5 mL of anhydrous DMF was prepared under N_2 atmosphere. To this mixture, a solution of 122 mg (109 μL , 1.39 mmol) of dipicolylamine was added dropwise. The mixture was magnetically stirred overnight at room temperature under a nitrogen atmosphere. The solvent was then removed under vacuum. The residue was partitioned between 20 mL each of water and chloroform. The aqueous phase was extracted three more times with 20 mL of CHCl_3 . The organic phases were combined and dried with Na_2SO_4 . The solvent was evaporated *in vacuo*. The residue was subjected to flash column chromatography (SiO_2 , 8:1:0.1 CHCl_3 :MeOH: NH_4OH). 124 mg (68% yield) of product was obtained. R_f : 0.64 (SiO_2 , 8:1:0.1 CHCl_3 :MeOH: NH_4OH). ^1H NMR (400 MHz, CDCl_3) δ ppm 1.25 (t, $J=7.15$ Hz, 3H) 2.11 (quin, $J=6.59$ Hz, 2H) 2.53 (t, $J=7.28$ Hz, 2H) 3.66 (s, 4H) 3.78 - 3.90 (m, 8H) 4.00 (t, $J=6.02$ Hz, 2H) 4.15 (q, $J=7.03$ Hz, 2H) 6.86 (s, 2H) 7.08 (s, 1H) 7.14 (t, $J=5.90$ Hz, 4H) 7.55 - 7.75 (m, 8H) 8.51 (d, $J=4.77$ Hz, 4H).

2.5.3.1.4 4-(3,5-Bis((bis(pyridin-2-ylmethyl)ammonio)methyl)phenoxy)-butanoate chloride (12)



127 μ L of 20% NaOH solution (w/w) was added dropwise to a solution of 120 mg (0.19 mmol) of **11** in 2 mL of THF, 1 mL of H₂O, and 1 mL of MeOH. The reaction was refluxed for 2 hours. After the reaction is complete, the reaction carefully neutralized with 0.1 M HCl. The solvent was removed *in vacuo*. 10 mL CHCl₃ was added to the residue, and the product was dissolved, NaCl crystals were precipitated. The CHCl₃ solution was collected from the mixture, and the liquid-solid extraction process was repeated two additional times. The solvent was removed *in vacuo* and 120 mg (0.18 mmol, 99% yield) of **12** was obtained. ¹H NMR (400 MHz, CDCl₃) δ ppm 1.91 (br s, 2H) 2.28 (br s, 1H) 3.44 (br s, 1H) 3.49 - 3.60 (m, 4H) 3.65 (br s, 1H) 3.71 (br s, 7H) 3.87 (br s, 2H) 6.73 - 6.85 (m, 2H) 6.90 (br s, 1H) 6.97 - 7.18 (m, 4H) 7.34 - 7.63 (m, 8H) 8.51 (br s, 4 H).

2.5.3.1.5 BDPA-Bearing Glass Microspheres



20 mg of BDPA-Acid (**12**) was dissolved in 1 mL of DMF at 0 °C. Subsequently, 5.9 mg (0.031 mmol) of PfpOH, 6.1 mg (0.031 mmol) of EDC, and 0.8 mg (0.006 mmol) of DMAP were added sequentially. The reaction mixture was stirred at 0 °C for 10 minutes before allowing it to reach room temperature. The reaction was left to proceed overnight. In a separate solution, 4.1 mg (5.41 μ L, 0.031 mmol) of DIPEA was dissolved in 0.5 mL of DMF and added to the reaction mixture. Then, 1 mg of GM-Amine were introduced into the reaction. The reaction was stirred at room temperature under N₂ atmosphere overnight. The solvent was removed under vacuum.

The resulting product, BDPA-GM-II, was washed three times with 25 mL of deionized water and once with 25 mL of ethanol. The glass microspheres were then dried under vacuum. Characterization was done with ATR.

CHAPTER 3

RESULTS AND DISCUSSION

3.1.1 Antimicrobial Photodynamic Therapy Experiments

1a-d molecules were synthesized by other student and **1d** was chosen for the studies of antimicrobial inactivation since **1d** contains an iodine atom in its *meso* position, the heavy atom effect would be greater compared to **1a**, **1b**, or **1c**. The heavy atom effect enhances intersystem crossing, leading to an increased singlet oxygen quantum yield. In order to identify the most desirable photosensitizer for antibacterial photodynamic inactivation and efficient photosensitization, **1d** was chosen for investigation against two Gram (+) bacteria (*Staphylococcus aureus* ATCC 25923 and *Bacillus cereus* ATCC 14579) and two Gram (-) bacteria (*Escherichia coli* Top10 and *Pseudomonas aeruginosa* ATCC 9027). The selection of these four bacterial strains was based on the existence of their variants, which exhibit single and multi-drug resistance. Gram (+) and Gram (-) bacteria display distinct cell wall compositions and permeability, resulting in different responses to various photosensitizers. Gram (+) bacteria possess peptidoglycan layers as their cell membrane and lack an outer membrane, rendering their membrane permeability less selective compared to Gram (-) cells. Gram (-) cells, on the other hand, have a thin peptidoglycan layer surrounded by lipopolysaccharide, which forms an outer membrane. It is known that Gram (+) bacteria are more susceptible to positively charged photosensitizers. Hence, both types of bacteria were examined in this study.

3.1.1.1 First Generation Approach for Antimicrobial Photodynamic Therapy

Zn₂BDPA-ligands selectively bind to bacterial cell walls due to two main factors: the ionic charge and the favorable coordination between Zn(II) and phosphate groups. Bacterial cell membranes carry a negative charge, while Zn₂BDPA-ligands possess a positive charge, facilitating the interaction. Furthermore, the geometric compatibility of Zn(II) and phosphate groups enhances the binding affinity. Both of these reasons depend on the presence of zinc on the BDPA-ligand. Due to this fact, in the first generation approach, there was an additional 20 μM Zn(NO₃)₂ in the bacterial suspension to have enough Zn(II) in the system to prevent the complex to disassociate upon dilution. DPA ligands are known to have a K_d (dissociation constant) value of 0.01 μM ¹¹⁷. Complete photoinactivation of 5x10⁶ CFU/mL of the Gram (-) bacterial species *E. coli* was observed at 2.5 μM concentration of **1d**. While **1d** exhibited dark toxicity, full inactivation was not observed for *E. coli* even at a 10 μM concentration of **1d** (Figure 17).

Complete photoinactivation of 5x10⁶ CFU/mL of the Gram (-) bacterial species *S. aureus* in the first-generation approach was observed at 0.5 μM concentration of **1d**. Full inhibition because of the dark toxicity was observed for *S. aureus*, which is different than in the case of *E. coli*. Dark toxicity was observed at the same concentration, 0.5 μM of **1d**, in which full photodynamic inactivation was also observed. However, since dark DMSO controls was not as expected, the experiments stopped and the problem of dark DMSO controls was investigated.

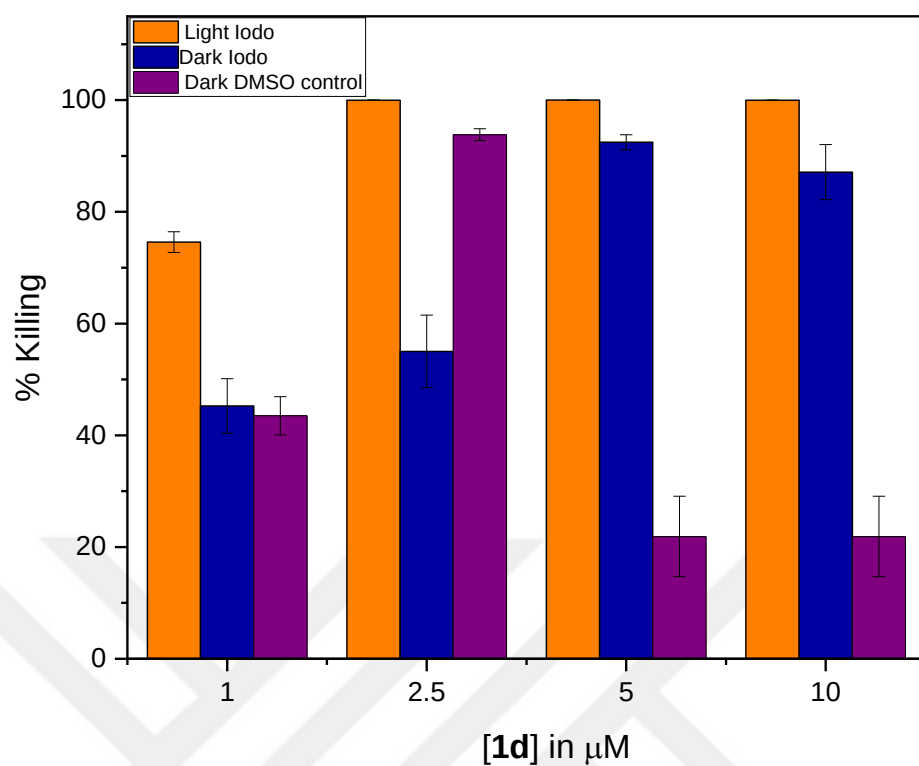


Figure 17. Photodynamic inactivation and dark toxicity of Iodo-Zn₂BDPA-porphyrin(**1d**) on *E. coli*. The error bars represent the standard deviation from at least three independent experiments.

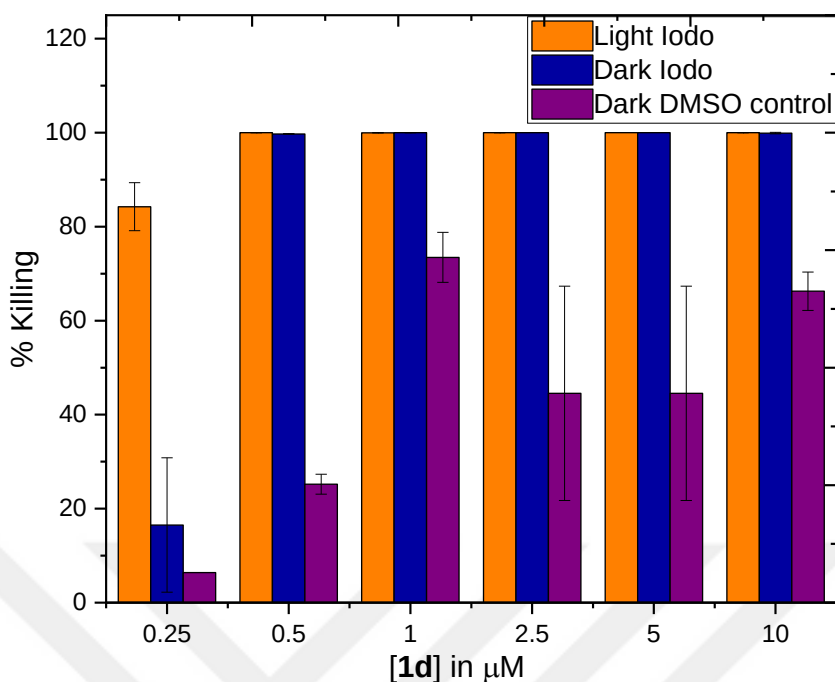


Figure 18. Photodynamic inactivation and dark toxicity of Iodo- Zn_2BDPA -porphyrin (**1d**) on *S. aureus*. The error bars represent the standard deviation from at least three independent experiments.

3.1.1.2 Zinc Toxicity on Bacterial Cells

Data in both *S. aureus* and *E. coli* experiments exhibited a similar issue. The dark DMSO control, which had the same composition as the light and dark samples except for the absence of photosensitizer, showed a lower bacterial count than expected. During the colony counting step, it was anticipated to count 500 bacteria on the LB-agar plate. However, DMSO controls exhibited bacterial cell inactivation ranging from 20% to 80%. It is known that DMSO can be toxic to bacterial cells at concentrations above 10%, but the bacterial suspensions only contained 1% DMSO. Only a constant volume of 15 μL was added to bacterial suspensions with a final volume of 1500 μL in all the samples, including light and dark samples and DMSO controls. Hence, DMSO alone could not account for the observed inactivation. Further investigation was conducted to determine the underlying cause. In order to investigate the root cause of the observed phenomenon, a thorough investigation was

undertaken. The focus shifted towards exploring the impact of various concentrations of $\text{Zn}(\text{NO}_3)_2$ on bacterial inactivation was examined. It was observed that 20 μM $\text{Zn}(\text{NO}_3)_2$ exhibited toxicity to bacterial cells. When looked in the literature, similar observation was reported.^{118,119} In the experiments, starting from 1 μM , there was a gradual increase in bacterial inactivation, with the trend continuing up to 20 μM for the Gram (-) bacterial species *E. coli* (Figure 19). This finding suggests a direct correlation between the concentration of $\text{Zn}(\text{NO}_3)_2$ and the level of bacterial inactivation, emphasizing the potential significance of this ion in influencing bacterial viability. Also, MIC screening test was performed and for *S. aureus*, MIC was found to be 1.25 mM and MBC was found out to be 10 mM. Therefore, in the second-generation approach, the decision was made to refrain from adding the excess $\text{Zn}(\text{NO}_3)_2$, which was added to keep the system saturated with Zn(II) so that the Zn_2BDPA complex was stable under dilution.

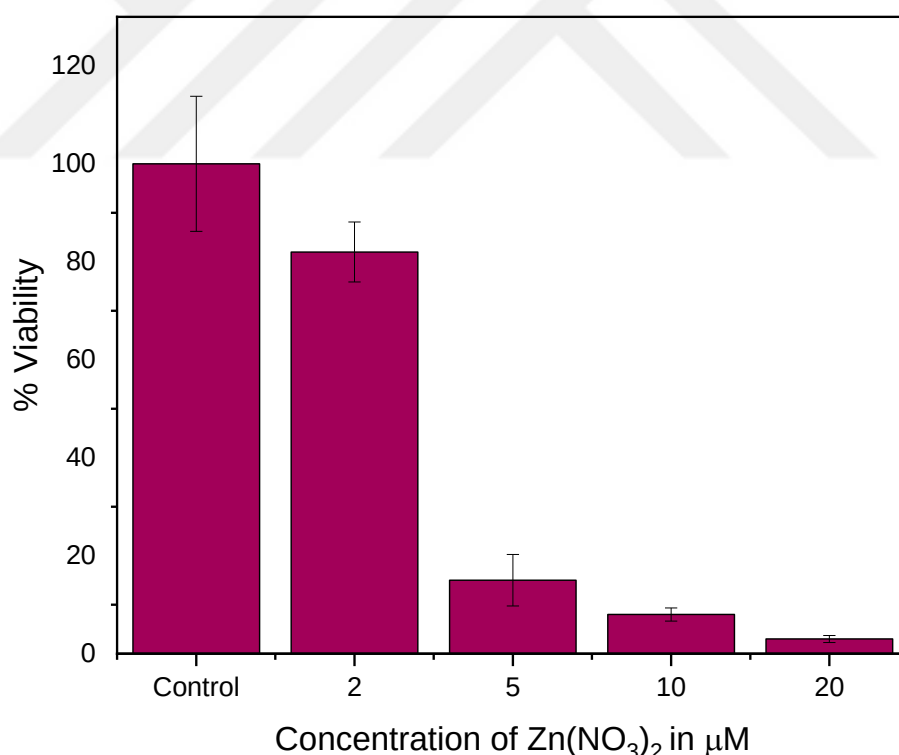


Figure 19. The effect of $[\text{Zn}(\text{NO}_3)_2]$ on *E.coli* Top10 viability. The error bars represent the standard deviation from at least three independent experiments

3.1.1.3 Second Generation Approach for Antimicrobial Photodynamic Therapy

Following the discovery of zinc toxicity on bacterial cells, the experiments were repeated with an updated procedure. The bacterial cell number was reduced to 1×10^6 CFU/mL, and the addition of excess zinc ions was omitted. The light and dark toxicity of **1d** in the 1-20 μ M concentration range was investigated against 1×10^6 CFU/mL suspensions of the aforementioned four bacterial species. Complete photoinactivation, corresponding to at least a log6 reduction in the number of viable bacterial cells, was observed for Gram (+) bacterial species *S. aureus* (Figure 20) and *B. cereus* (Figure 21) at 5 μ M **1d** concentration. Complete photoinactivation at 5 μ M **1d** concentration was also observed for the Gram (-) bacterial species *E. coli* (Figure 22), but not for the Gram (-) *P. aeruginosa* (Figure 23).

Even at a concentration of 20 μ M **1d**, approximately 2.5% of *P. aeruginosa* cells were able to survive upon irradiation. This outcome can be a result of the exceptional ability of *P. aeruginosa* to form biofilms, which act as protective barriers that hinders the penetration of antibiotics and antimicrobial photodynamic therapy agents into the bacterial cells.¹²⁰ The failure to reach complete photoinactivation in these studies could be related to residual biofilm that remain on the surfaces of the cells even after washing with buffer. The investigation of higher concentrations of **1d** for photoinactivation of *P. aeruginosa* was not possible because of the low solubility of the photosensitizer **1d**, as evidenced by the precipitation of **1d** at concentrations above 20 μ M. Additionally, the literature supports the concept that *P. aeruginosa* exhibits greater resistance to photoinactivation compared to other bacterial species. A log7 reduction in viable cells of *S. aureus* and a log5 reduction in *C. albicans* was reported in a study, whereas *P. aeruginosa* exhibited only a log1 reduction under similar conditions.¹²¹ Similarly, Banfi *et al.* found that *P. aeruginosa* required higher concentrations of photosensitizer (7-35 μ M) compared to *E. coli* and *S. aureus* (1-3 μ M) for lethality.¹²² Machado *et al.* also reported that complete photoinactivation of *P. aeruginosa* required 40 μ M of photosensitizer.¹²³ These findings collectively

support the robustness and higher resistance of *P. aeruginosa* towards photoinactivation compared to other bacterial strains observed in this study.

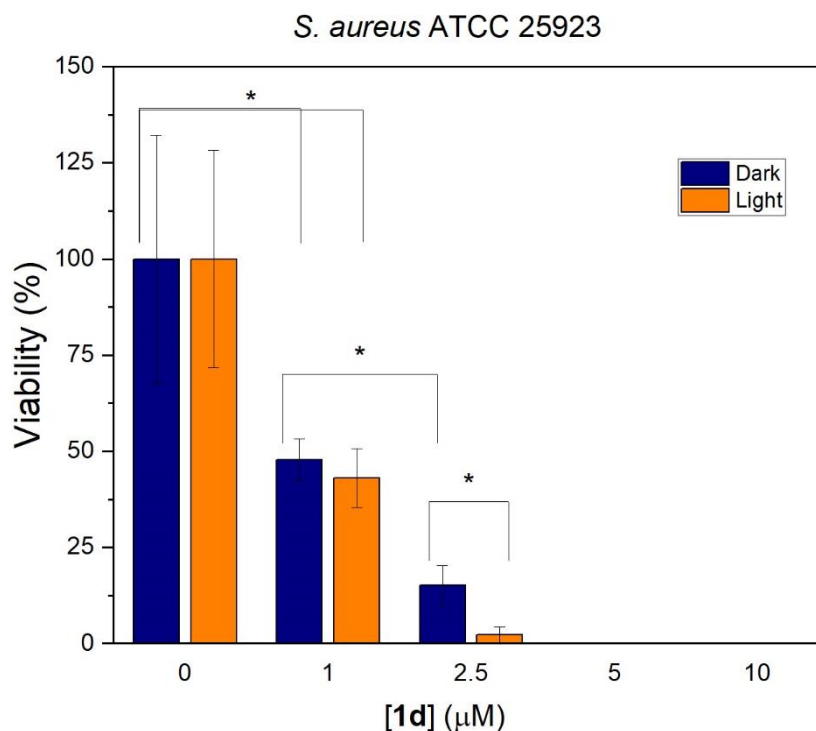


Figure 20. Concentration-dependent light and dark toxicities of Zn₂BDPA-porphyrin derivative **1d** against Gram (+) bacterial species *S. aureus*. The error bars represent the standard deviation from at least three independent experiments. An asterisk indicates a statistically significant difference at $p \leq 0.05$.

The aPDT agent **1d** evaluated in this study demonstrates favorable photoinactivation capability compared to other agents designed with a similar objective. Rice *et al.* investigated the targeted antimicrobial photodynamic inactivation of *E. coli* and *S. aureus* using a zinc(II) dipicolylamine BODIPY conjugate at a concentration of 10 μM, achieving 99.9% and 99.99% inactivation, respectively.³⁰ Zoltan *et al.* explored the photoinactivation of Zn-metallated porphyrins against *E. coli* and observed 50% inactivation.¹²⁴ Li *et al.* studied the photoinactivation of octacationic zinc phthalocyanines with 1,2-ethanediamine groups and their quaternized derivatives

against *E. coli*, achieving a log3 reduction in viable cell count at 5 μ M and a log5 reduction at 20 μ M.¹²⁵ Feng *et al.* synthesized Ruthenium(II) polypyridyl complexes with peripheral quaternary ammonium structures and demonstrated selective photoinactivation against *S. aureus*, achieving a log6 reduction in viable cell count at 15 μ M concentration.¹²⁶ Li *et al.* investigated the selective photodynamic inactivation of *B. subtilis*, *S. thermophilus*, *S. aureus*, *E. coli*, and *P. aeruginosa* cells using crystal violet (CV) and monopiperazine modified crystal violet (MPCV) at concentrations of 5 μ M and 10 μ M. They achieved a log6 reduction in viable cell count against *E. coli* at 10 μ M for both CV and MPCV but not against the other strains.¹²⁷ Zhou *et al.* designed a polyarginine chlorophyll derivative (PA7) and evaluated its photoinactivation effect against *S. aureus* and *E. coli*. The survival rate of *S. aureus* was around 5% at 5 μ M PA7, while it was approximately 55% for *E. coli* at the same concentration.¹²⁸ Oliveira *et al.* reported that cationic porphyrins, including Tri-Py⁺-Me-COOH, toluidine blue O, Tri-Py⁺-Me-Ph, and Tetra-Py⁺-Me, achieved reductions of 6.5, 6.4, 5.7, and 5.3 log units in viable cell count, respectively, at a concentration of 60 μ M against *B. cereus*.¹²⁹

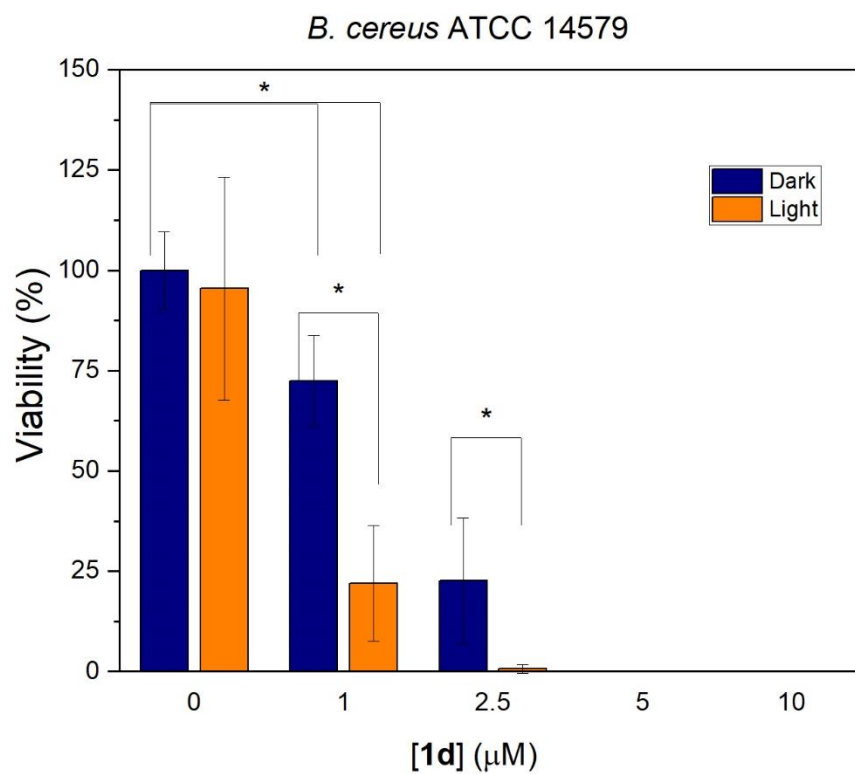


Figure 21. Concentration-dependent light and dark toxicities of Zn₂BDPA-porphyrin derivative 1d against Gram (+) bacterial species *B. cereus*. The error bars represent the standard deviation from at least three independent experiments. An asterisk indicates a statistically significant difference at $p \leq 0.05$.

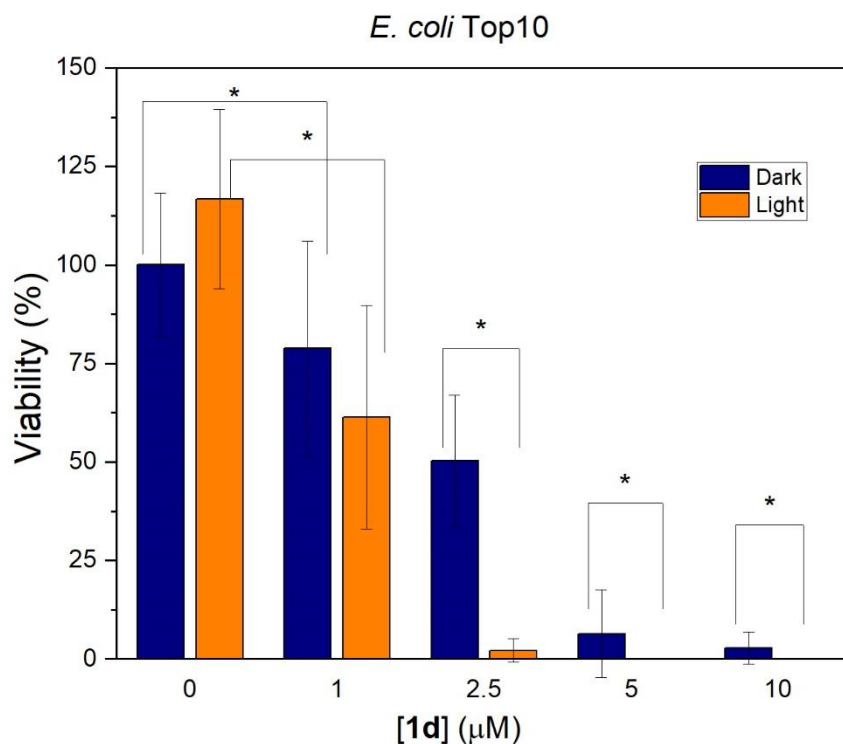


Figure 22. Concentration-dependent light and dark toxicities of Zn₂BDPA-porphyrin derivative **1d** against Gram (-) bacterial species *E. coli*. The error bars represent the standard deviation from at least three independent experiments. An asterisk indicates a statistically significant difference at $p \leq 0.05$.

In comparison to the previous studies that were reported in the literature, the aPDT agent **1d** developed in this study demonstrates competitive photoinactivation capability against Gram (+) bacterial species *S. aureus* and *B. cereus*, achieving complete inactivation at 5 μM concentration. Furthermore, it exhibits effective photoinactivation against the Gram (-) bacterial species *E. coli*, although incomplete inactivation is observed at higher concentrations.

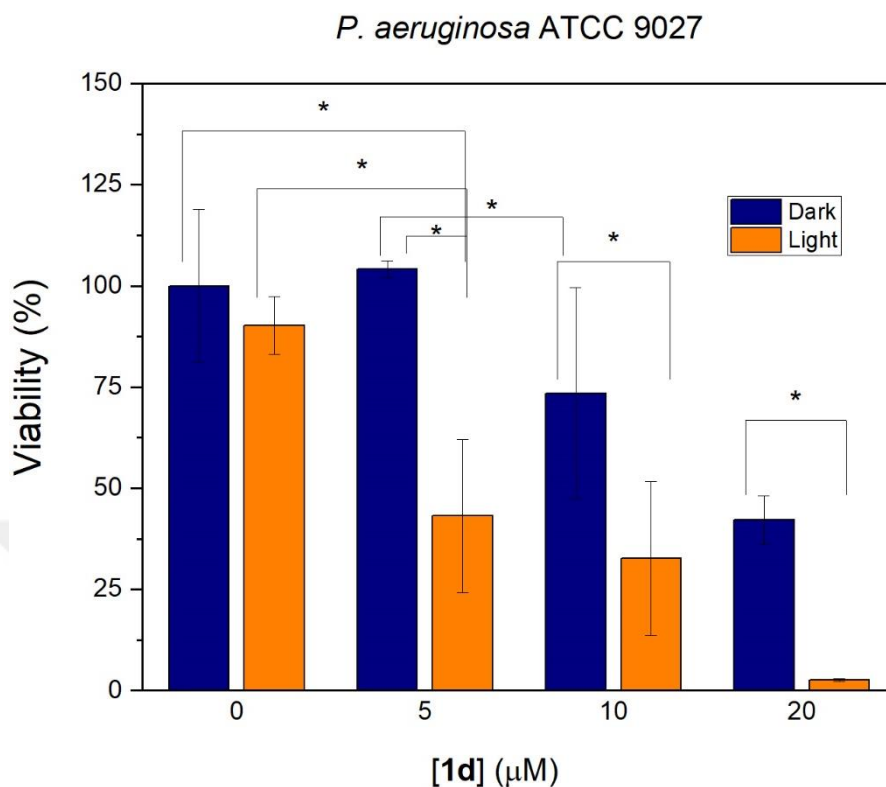


Figure 23. Concentration-dependent light and dark toxicities of Zn₂BDPA-porphyrin derivative **1d** against Gram (-) *P. aeruginosa*. The error bars represent the standard deviation from at least three independent experiments. An asterisk indicates a statistically significant difference at $p \leq 0.05$.

In addition to its photodynamic inactivation effect, the aPDT agent **1d** also exhibited significant dark toxicity. The data presented in Figure 20 and Figure 21 suggest that Gram (+) bacterial species are more susceptible to the dark toxicity of **1d** compared to Gram (-) bacterial species, particularly at the concentration of 2.5 μM. Under irradiation, only approximately 2% of *S. aureus*, *B. cereus*, and *E. coli* cells survived at 2.5 μM **1d**, while in the dark conditions, approximately 20% of *S. aureus* and *B. cereus* cells survived compared to 50% of the cells for *E. coli* at dark at 2.5 μM concentration. *P. aeruginosa* cells exhibited the least susceptibility to the dark toxicity of **1d**. It should be noted that Zn₂BDPA complexes, to which **1d** belongs,

are known to be non-toxic to bacterial or mammalian cells.¹³⁰ Therefore, the observed dark toxicity of **1d** could be attributed to the porphyrin moiety it contains. Porphyrins are known to exhibit dark toxicity against bacterial cells. Sălăgeanu *et al.* conducted disk diffusion assays using various concentrations of porphyrins against different bacterial strains in the dark and reported their dark toxicity.¹³¹ Gyulkhandanyan *et al.* also reported that porphyrins have dark toxicity as well as photo-activated toxicity against bacterial strains.¹³² It is also reported that porphyrins show dark toxicity of log5 reduction at 1 and 10 μM ¹³³ and 100% inhibition 1-70 μM .¹³⁴

Moreover, it is known that Gram (+) and Gram (-) bacteria differ in their cell permeabilities due to variations in their cell membrane structures. Gram (+) bacteria have outer layers of peptidoglycan and lipoteichoic acid, which result in a less effective and more porous barrier. This allows photosensitizers to diffuse to the target sites within the cell more easily. In contrast, Gram (-) bacteria have an asymmetric bilayer of lipopolysaccharides (LPS) and phospholipids, which impede the diffusion of photosensitizers to the cell membrane. These structural differences between Gram (+) and Gram (-) bacterial cells may explain the enhanced dark toxicity of **1d** against Gram (+) bacterial species observed in this study.

To investigate the structural origin of the dark toxicity observed for **1d**, different fragments of **1d** and a similar derivative without the iodine atoms (**1a**) were selected and their bacterial inactivation effects were investigated. Porphyrin fragments selected were metalated amino-tetraphenylporphyrin derivative **13** and metalated amino-(iodo)-tetraphenylporphyrin derivative **14** (Figure 25). **14** was selected to mimic the porphyrin portion of **1d**. To mimic the Zn_2BDPA portion of **1d**, complexes **15** and **Zn₂-10** were selected (Figure 25). It was important to see whether these 2 complexes had any inhibitory effect on bacterial cells at the concentration levels relevant to this work. The experimental procedure was the same as described in the experimental section for broth microdilution experiments, the dark and light controls were also the same, and the concentration range was also the same as in previous experiments. For **1a**, the bacterial inactivation both in the dark and in the presence

of light was the same as **1d**. MIC of molecules **13** and **14** was greater than 10 μM for both dark and light controls. For **15** and **Zn₂-10** were light control experiments were not done. These complexes did not show any toxicity against *S. aureus*. Therefore, it can be concluded that the dark toxicity of **1d** is likely not due to the any subgroups that **1d** bears, but rather is a result of synergistic interactions between them (Table 5). It may be possible that the observed dark toxicity is due to membrane disruption. These porphyrin systems are known to bind bacterial cell membranes. Therefore, the binding of porphyrin onto the cell membrane can cause disturbances in the membrane that result in bacterial cells being in stress and eventually die. The confocal microscopy image shows that **1a** binds to the bacterial cells. The left side of the image shows the GFP expressing *E. coli* K12 strain. The middle picture shows the location of porphyrins, colored in red. The third image is the overlay image of the first two.

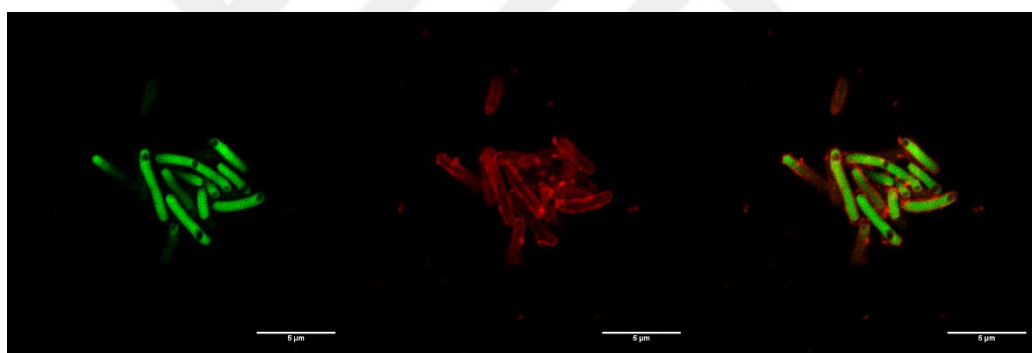


Figure 24. The confocal microscopy analysis of GFP expressing *E. coli* cells treated with **1a** (Zeiss LSM 800 at 63x magnification).

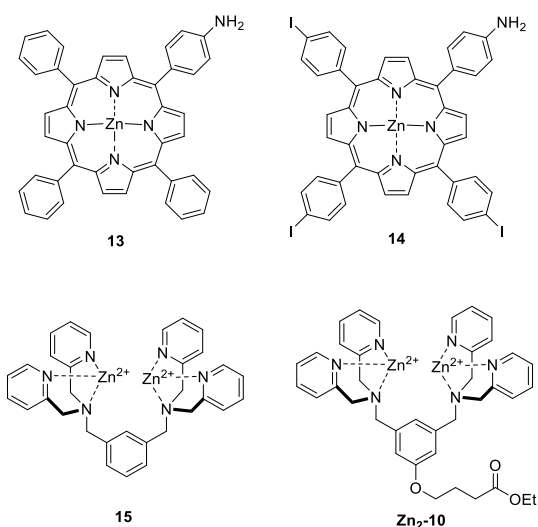


Figure 25. Fragments for which light and/or dark toxicity was investigated using the broth microdilution method.

Table 5. Light and dark toxicity values of molecules investigated in this study against *S. aureus* ATCC 25923.

Molecule	Light Toxicity (MIC, μ M)	Dark Toxicity (MIC, μ M)
Zn ₂ BDPA-(H)-Porphyrin (1a)	5	5
13	>10	>10
14	>10	>10
15	-	>10
Zn ₂ -10	-	>10

3.2 Broth Microdilution Experiments for Antibiotic Efficacy Determination

In preparation for future liposomal encapsulation studies and to also potentially simplify and accelerate aPDT efficacy studies, it was decided to investigate the broth microdilution technique for MIC determination and to establish appropriate protocols. In broth microdilution experiments, Clinical and Laboratory Standards Institute (CLSI) standards were followed, and appropriate procedures were

implemented. In order to understand if the procedures that were designed were reproducible, standard antibiotics were used to obtain MIC values and these values were compared to the values given in the CLSI reference source and elsewhere in the literature. Rifampin, ciprofloxacin, vancomycin and amoxicillin were applied to these strains: *P. aeruginosa* ATCC 9027, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *B. cereus* ATCC 14579, *B. subtilis* ATCC 6633. The objective was to obtain the same MIC values as reported and the that objective has been achieved. The broth microdilution procedures established in this study are thus valid. It should also be noted that, it is highly recommended that whenever broth microdilution experiments are performed with new materials, an antibiotic reference as a positive control should always be used. Therefore, it was important that all of the antibiotic controls have the same MIC value as reported in the literature or reported by CLSI. In this study, ciprofloxacin was observed to have a MIC value of 0.75 µg/mL against *P. aeruginosa* ATCC 9027 and a MIC value of 0.24 µg/mL against *P. aeruginosa* ATCC 27853 (Table 6 and 7).¹¹⁶ In our study rifampin has a MIC value of 16 µg/mL against *P. aeruginosa* ATCC 27853 (Table 7). Against *S. aureus* ATCC 25923 rifampin had a MIC value of 0.004 µg/mL, vancomycin had a MIC value of 1-2 µg/mL, ciprofloxacin had a MIC value of 0.24 µg/mL, and amoxicillin had a MIC value of 0.0375 µg/mL (Table 8).^{116,135,136} Against *E. coli* ATCC 25922, in this study. ciprofloxacin, amoxicillin, and vancomycin had MIC values of 0.0075-0.015, 4, and 128 µg/mL, respectively (Table 9).^{116,137,138} It was observed in this study that vancomycin had a MIC values of 0.125 µg/mL and 1 µg/mL against *B. subtilis* ATCC 6633 and *B. cereus* ATCC 14579, respectively (Table 10 and 11).¹³⁹ Rifampin had a MIC value of 0.12 µg/mL against *B. subtilis* ATCC 6633 (Table 10).¹⁴⁰ These values are all the same as reported in the literature.

Table 6. Antibiotic MIC value observed for *P. aeruginosa* ATCC 9027 through broth microdilution.

<i>P. aeruginosa</i> ATCC 9027	MIC (µg/mL)
Ciprofloxacin	0.75

Table 7. Antibiotic MIC values observed for *P. aeruginosa* ATCC 27853 through broth microdilution.

<i>P. aeruginosa</i> ATCC 27853	MIC (µg/mL)
Rifampin	16
Ciprofloxacin	0.24

Table 8. Antibiotic MIC values observed for *S. aureus* ATCC 25923 through broth microdilution.

<i>S. aureus</i> ATCC 25923	MIC (µg/mL)
Rifampin	0.004
Vancomycin	1-2
Ciprofloxacin	0.24
Amoxicillin	0.0375

Table 9. Antibiotic MIC values observed for *E. coli* ATCC 25922 through broth microdilution.

<i>E. coli</i> ATCC 25922	MIC (µg/mL)
Ciprofloxacin	0.0075-0.015
Amoxicillin	4
Vancomycin	128

Table 10. Antibiotic MIC values observed for *B. subtilis* ATCC 6633 through broth microdilution.

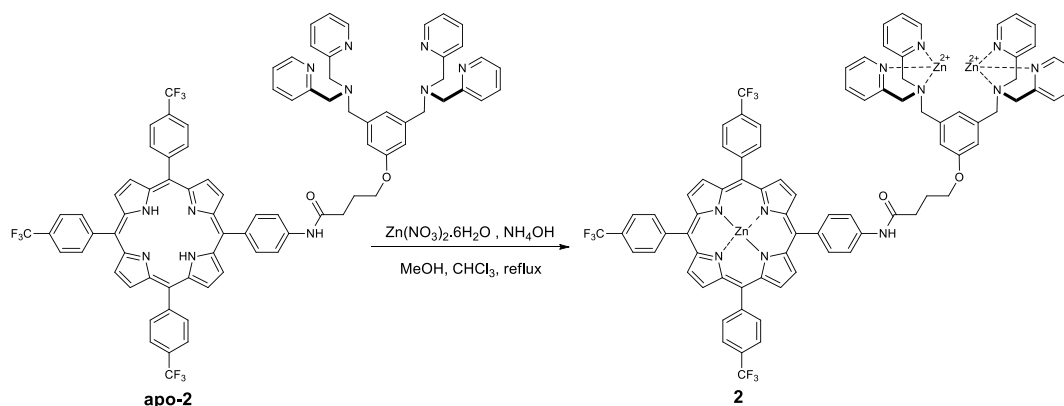
<i>B. subtilis</i> ATCC 6633	MIC (µg/mL)
Rifampin	0.12
Vancomycin	0.125

Table 11. Antibiotic MIC values observed for *B. cereus* ATCC 14579 through broth microdilution.

<i>B. cereus</i> ATCC 14579	MIC ($\mu\text{g/mL}$)
Vancomycin	1

3.2.1 Broth Microdilution Experiments for Antibacterial Photodynamic Inactivation Efficacy Determination

Liposomal encapsulation of drugs increases the efficacy of drugs on the target and decreases systematic toxicity. Thus, if a porphyrin or an antibiotic is encapsulated with liposomes that have targeting groups on their outer surface, the bacterial inactivation efficacy of porphyrins or antibiotics can be increased. For the encapsulation of porphyrins, molecule **2** ($\text{Zn}_2\text{BDPA-CF}_3\text{-Porp}$) was chosen since it was thought that the $-\text{CF}_3$ groups would favorably interact with the lipids of liposomes and can thus integrate into the lipid bilayer. Metalation of **apo-2** (BDPA- CF_3 -Porp) was conducted before any encapsulation since BDPA ligands should be in complex form with Zn(II) in order to interact with phosphate groups on the outer cell membrane of the bacterial cells. Metalation of **apo-2** to give **2** was accomplished by refluxing **apo-2** with $\text{Zn(NO}_3)_2$ in the presence of ammonia (Scheme 5).



Scheme 5: Metalation of **apo-2** to give metaloporphyrin derivative **2**.

Metalation of the porphyrin was demonstrated through the unique absorption spectra of porphyrins (Figures 27 and 28). When porphyrin is not metalated, it is called “free base form”, when it is metalated it is called “metalloporphyrin”. In their free base form, porphyrins have a Soret band in between 380-500 nm depending on whether the porphyrin is β - or *meso*-substituted. This Soret band is due to the transition from the ground state to the second excited state ($S_0 \rightarrow S_2$). Q bands are in the second region of the spectra and when the porphyrin in its free base form, four Q bands are shown. They are between 500-750 nm, and they are due to the weak transition to the first excited state ($S_0 \rightarrow S_1$). When the relative intensities of Q bands are such that $IV > III > II > I$, the spectrum then is *etio*-type and porphyrins called *etioporphyrins*. The first two of them are called Qy_1 and Qy_2 , and the other two are called Qx_1 and Qx_2 . The subscript number represents the orientation of the vector of the absorbed light (Figure 26).

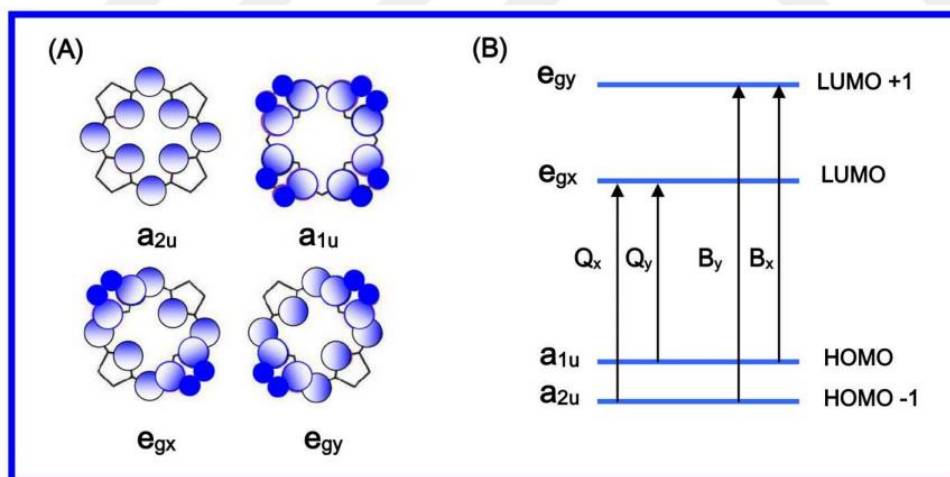


Figure 26. Porphyrin HOMOs and LUMOs. (B) shows the Q band and Soret(B) band transitions.¹⁴¹

When a porphyrin is metalated, there are two Q bands in the spectrum. The Q bands are called α and β . The intensities of these bands are related to the stability of the metal complex. If α is greater than β , it means that metal complex is square-planar.

Since symmetry is established after metalation, there still remains two Q bands Q_y and Q_x (Figure 28).^{141,142}

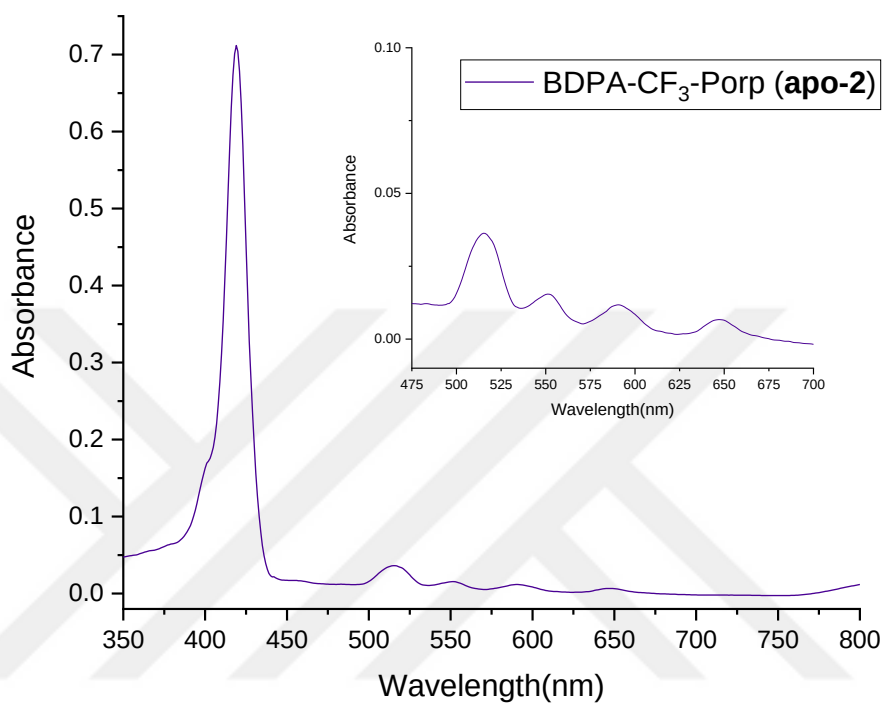


Figure 27. UV-Vis spectrum of **apo-2**. (insert Q bands)

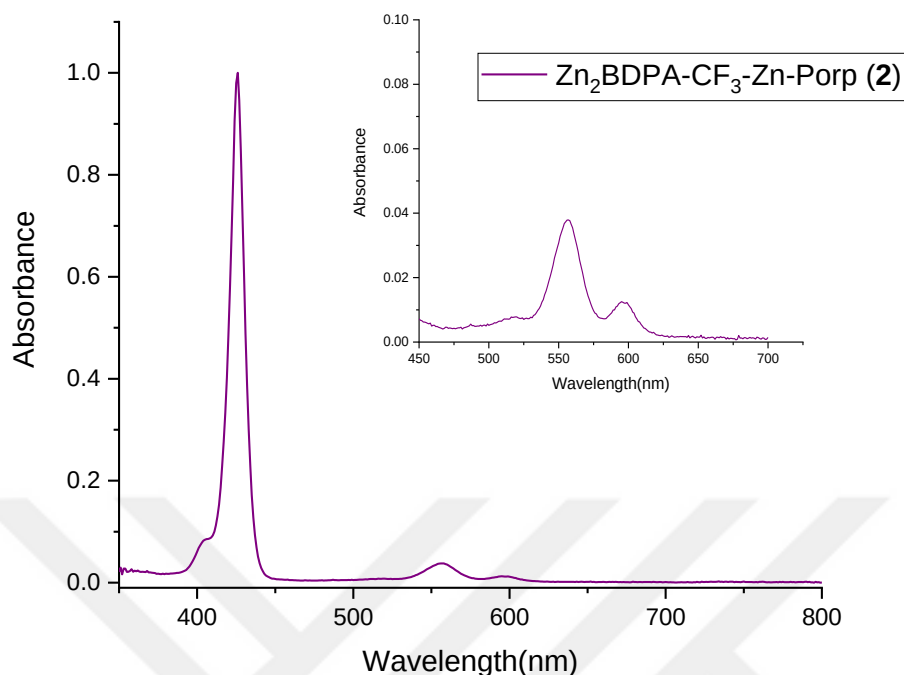


Figure 28. UV-Vis spectrum of **2**. (insert Q bands)

After metalation of the porphyrin, antimicrobial photodynamic inactivation efficacy of **2** was investigated. To validate the applicability of the broth microdilution technique to antibacterial photodynamic inactivation (aPDI) efficacy determination, aPDI against *E. coli* was done using **1d** and the same efficacy was observed as with the second generation aPDI experiments done using the colony counting method. Thus, the broth microdilution approach has been validated for aPDI efficacy determination. This method was applied to determine the aPDI efficacy of **2** against various bacterial strains and the results are given in Table 12.

Table 12. MIC values of **2** against various bacterial species in the presence and absence of light.

Bacterial Strain	Light Toxicity (MIC, μM)	Dark Toxicity (MIC, μM)
<i>E. coli</i> ATCC 25922	5	>10
<i>S. aureus</i> ATCC 25923	2.5	2.5
<i>B. cereus</i> ATCC 14579	5	5

<i>P. aeruginosa</i> ATCC 9027	>20	>20
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Light toxicity MIC value for **2** against *E. coli* was found to be 5 μM and dark toxicity MIC was higher than 10 μM . Against the other Gram (-) bacterial strain *P. aeruginosa*, **2** had a MIC value which was higher than 20 μM ; however, unfortunately the solubility problems of porphyrins in buffer limits the concentration range which could be screened to 20 μM and lower. At higher concentrations **2** precipitated out. Against the Gram (+) bacterial strain *B. cereus*, **2** had both light and dark toxicity MIC values of 5 μM . *S. aureus* was most susceptible against **2**, where **2** had both dark and light toxicity MIC values of 2.5 μM .

In order to increase bacterial inactivation efficacy, liposomal encapsulation of **2** was attempted. At first, formulation **Lipo-1** was established where the liposomes would have a lipid composition of 2 mol% $\text{Zn}_2\text{BDPA-CF}_3\text{-Porphyrin (2)}$ /POPC/Chol (2/68/30, mol/mol/mol), with 3.32 μmol total lipid per 1 mL of liposomal dispersion. After extrusion, DLS data was taken with Zetasizer Nano. The average diameter was found to be 187.5 nm, with a PDI (polydispersity index) of 0.205 (Figure 29). When zeta potential was measured, the surface charge was found to be -2.92 mV, which was unexpected because Zn_2BDPA on the lipid surface was expected make the charge positive. Therefore, a new formulation was designed.

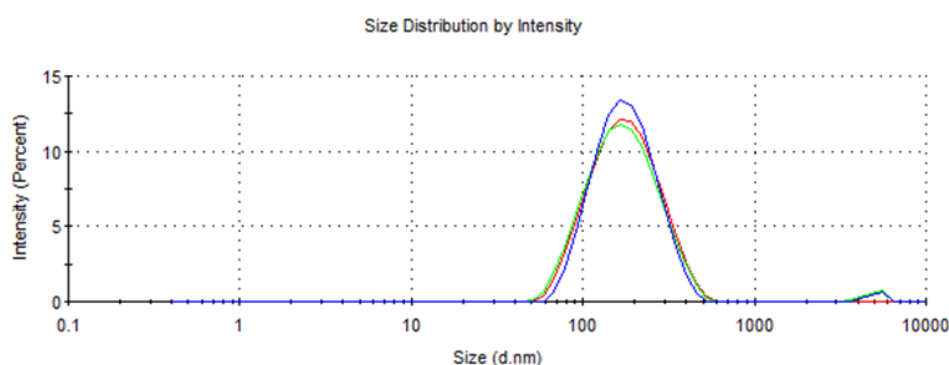


Figure 29. DLS size distribution graph for the first liposomal formulation (**Lipo-1**) for the encapsulation of **2**.

The second formulation (**Lipo-2**) contained only POPC (98 mol %) and 2 mol % of **2**. DLS measurements gave an average diameter of 176.3 nm, a PDI of 0.166, and a zeta potential of +2.92 mV (Figure 30). However, when the aPDI efficacy of this formulation was tested on bacterial cells, it did not exhibit any dark or light toxicity at concentrations where free **2** was found to be active. Thus, it was decided to increase the concentration of **2** in new liposomal formulations.

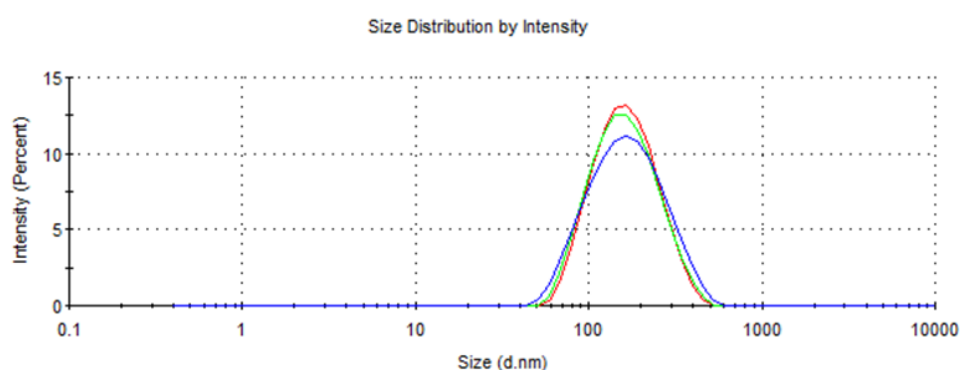


Figure 30. DLS size distribution graph for the second liposomal formulation (**Lipo-2**) for the encapsulation of **2**.

The third formulation (**Lipo-3**) contained 5 mol% Zn₂BDPA-CF₃-Porphyrin (**2**) and 95 mol% POPC, with 3.32 μ mol total lipid per 1 mL of liposomal dispersion. After extrusion, DLS and zeta potential measurements indicated that this formulation had liposomes with an average diameter of 156.6 nm, PDI of 0.114, and zeta potential of +7.07 mV (Figure 31). Although the percentage of targeting group per liposome was increased, still no aPDI effect was observed. Liposome formulations will be optimized in future studies until an aPDI effect is observed with these types of liposomes.

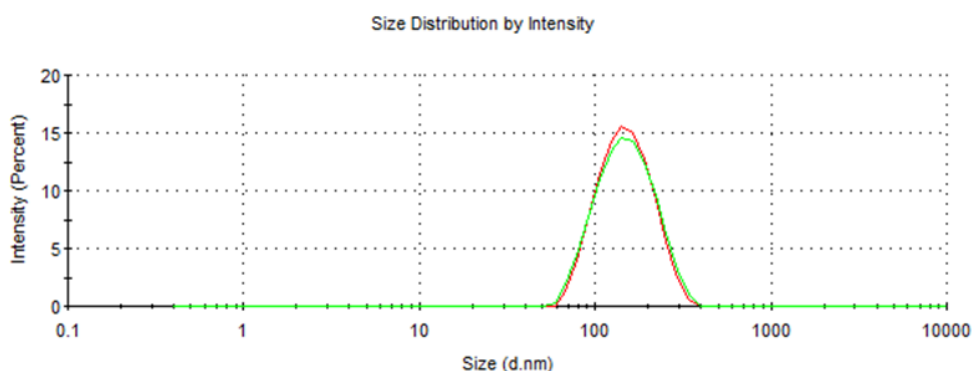


Figure 31. DLS size distribution graph for the third liposomal formulation (**Lipo-3**) for the encapsulation of **2**.

To ensure the encapsulation of porphyrins into liposomes, membrane dialysis experiment was performed. Two cells, separated by a 50 nm etch track filter, were present in the dialysis membrane. One cell was filled with buffer, while the other cell was filled with a liposomal dispersion. It was expected that after some time, free porphyrin would pass through the filter to the buffer side. However, the distinct purple color of porphyrins was not observed in the buffer, indicating that the porphyrins did not migrate to the buffer side. This suggests that the porphyrins are contained within the liposomes rather than simply interacting with them.

3.3 Rifampicin Liposomes

As a prototype of Zn₂BDPA-targeted liposomal antibiotics, it was decided to develop a liposomal formulation of the antibiotic rifampin. The targeting element used was a Zn₂BDPA conjugate of cholesterol (Zn₂BDPA-Chol), synthesized by another student. The formulation was Rifampin/Zn₂BDPA-Chol/POPC (10/5/85 mol/mol/mol), with 10 µmol total lipid in 1 mL liposome dispersion to give 1 mM rifampin in the dispersion. The average diameter of liposomes was found to be 154.1 nm, PDI was 0.088, and the zeta potential was measured at +6.02 mV (Figure 32). This formulation was expected to be more effective than free rifampicin, however,

they had the same MIC values. New liposomal formulations will be prepared and tested in future studies.

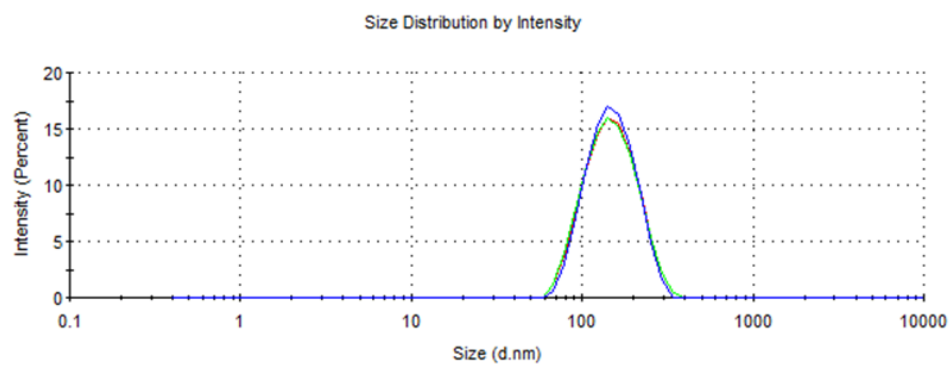
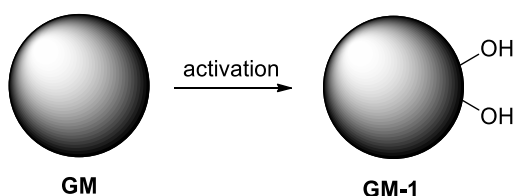


Figure 32. DLS size distribution graph for a liposomal formulation of rifampicin.

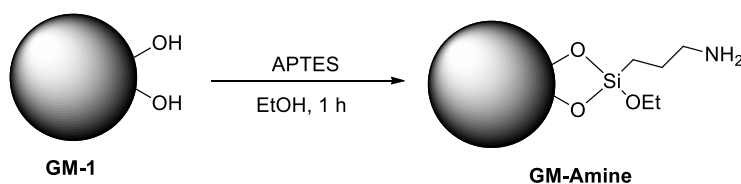
3.4 Studies Towards Zn₂BDPA Complex Bearing Glass Microspheres for the Sequestration of Bacterial Cells

3.4.1 Activation of Glass Microspheres



We aimed to derivatize the hydroxyl groups on the surface of the glass microspheres. Efficient derivatization of said groups requires removal of any contaminants. This removal of contaminants and increase of available surface hydroxyl groups is called “activation” of glass surfaces. In this study activation of glass microspheres (i.e., GMs) was done using piranha solution (i.e., mixture of H₂SO₄ and H₂O₂). After addition to piranha solution, the spheres were heated, and excess piranha solution was neutralized with NaOH. Supernatant was discarded and spheres were washed with DI water then ethanol. Activated glass microspheres were dried at 60 °C.

3.4.2 Amine-Derivatization of Activated Glass Microspheres Using APTES



After activation, derivatization of the surface of the GMs to display reactive and derivatizable primary amines silanization was performed. These primary amines could be used to conjugate BDPA ligands. To this end, activated glass microspheres were treated with APTES in ethanol. Characterization with ATR was unfortunately not very effective. Thus, an alternative way of characterization, the Kaiser test was

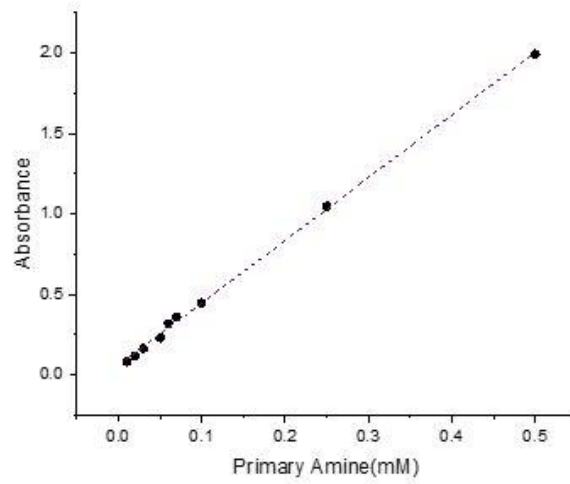


Figure 34. Calibration curve for the Kaiser test (Slope: 3.8975; Intercept: 0.056; R^2 : 0.999).

3.4.3 Calculation of Surface Amine Group Density of GMs after APTES Treatment Using the Kaiser Test

$$y = 3.8975x + 0.056$$

$$= \frac{(0.157 - 0.056)}{3.897} = 0.0259 \text{ mM}$$

$$\frac{0.0259 \text{ mM}}{0.002 \text{ L}} = 5.183 \times 10^{-5} \text{ mmol}$$

$$1 \text{ sphere has a volume of: } \frac{4}{3} \times 3.14 (0.01 \text{ cm})^3 = 4.18 \times 10^{-6} \text{ cm}^3$$

$$\frac{200 \text{ mg spheres}}{2500 \text{ mg/cm}^3} = 0.083 \text{ cm}^3$$

$$\frac{0.083 \text{ cm}^3}{4.18 \times 10^{-6} \text{ cm}^3} = 19138 \text{ spheres}$$

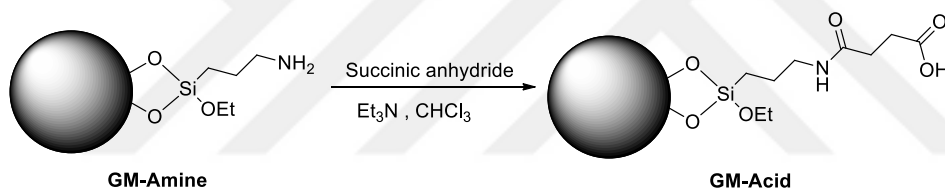
$$\frac{5.183 \times 10^{-5} \text{ mmol}}{19138 \text{ spheres}} = 2.7 \times 10^{-9} \text{ mmol}$$

$$2.7 \times 10^{-12} \text{ mol} \times 6.02 \times 10^{23} \text{ molecules/mol} \\ = 16.3 \times 10^{11} \text{ amine molecules}$$

$$\frac{16.3 \times 10^{11} \text{ amine molecules}}{4 \times \pi \times (1000 \text{ nm})^2} = 13 \text{ amine molecules/nm}^2$$

These calculations indicated that there were approximately 13 amine groups per square nanometer, which is much higher than the theoretical maximum of 6 amine groups per square nanometer. This observation may be due to irregularities at the surfaces of GMs, or some of the GMs may be hollow and the interior might have been derivatized as well. This question will be further addressed in future studies.

3.4.4 Reaction of GM-Amine with Succinic Anhydride



Scheme 7: Reaction of GM-Amine with succinic anhydride

For derivatization Strategy II, carboxylic acid group bearing glass microspheres were needed. Therefore, GM-Amine spheres were treated with succinic anhydride in chloroform. After washing with ethanol, spheres were dried at 60 °C. For characterization, ATR spectra were taken for both GM-Amine and GM-Acid. Unfortunately, there were no specific peaks for GM-Amine identifiable in the IR spectra. However, when GM-Amine were transformed into GM-Acid, three new peaks appeared. The peaks located at 1759 cm⁻¹, 1644 cm⁻¹, and 1450 cm⁻¹ show the presence of C=O (carbonyl), C=O (amide), and C–N bonds, respectively. The peak located at 1547 cm⁻¹ indicates the presence of NH bond. The peaks located at 1030 cm⁻¹ and 770 cm⁻¹ are indicative of Si–O–Si and Si–O bonds (Figure 35).[138]

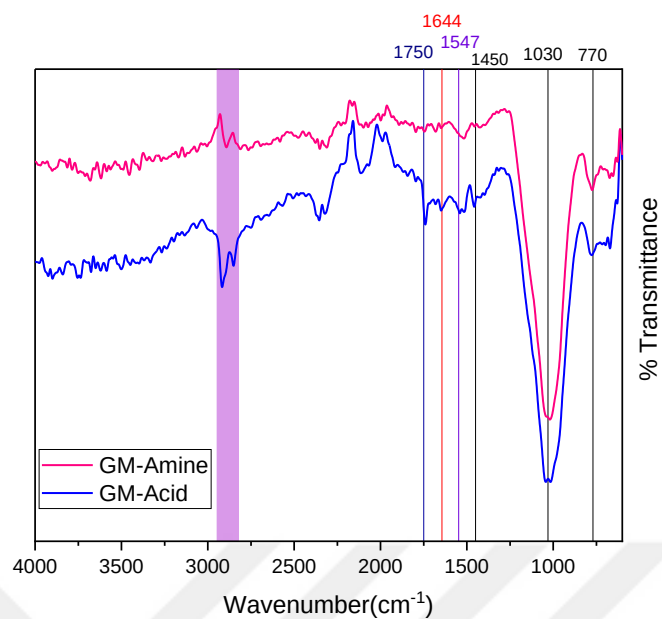
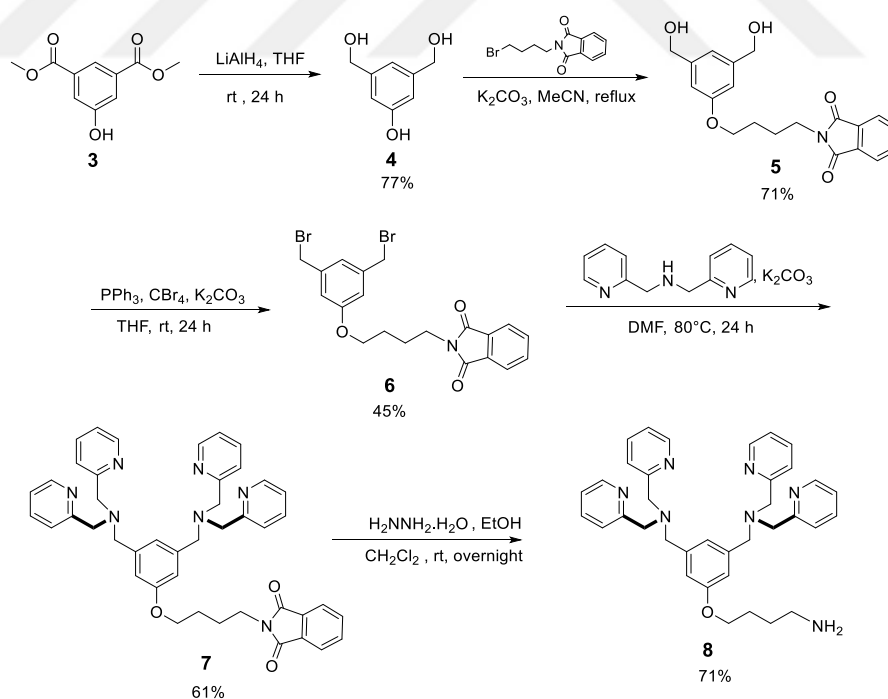


Figure 35. ATR-FTIR spectra of GM-Amine and GM-Acid.

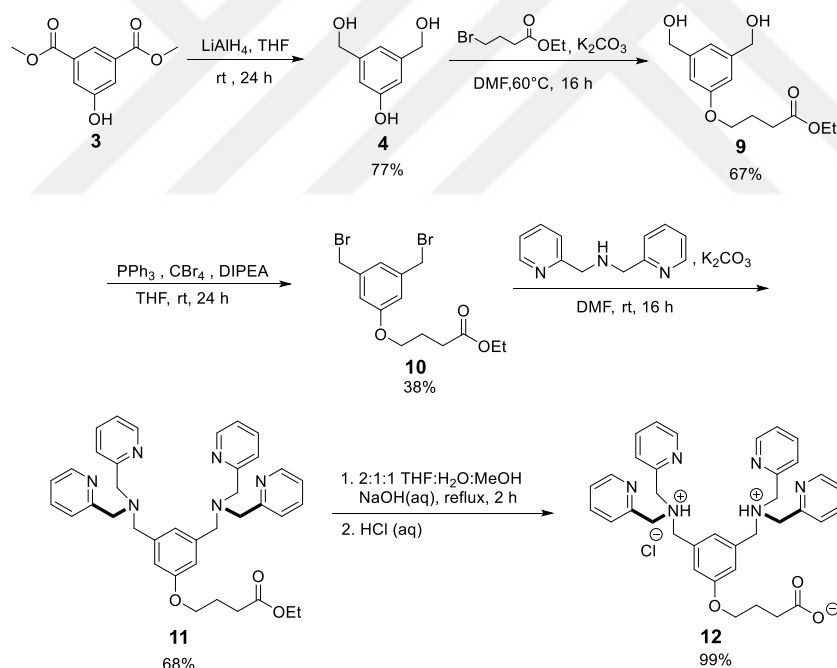
3.4.5 Synthesis of BDPA-Amine Ligand (8)



Scheme 8: Preparation of BDPA-Amine (8).

BDPA-amine ligand (**8**) could be conjugated GM-Acid. The synthesis of ligand **8** was carried out following a previously reported procedure and the synthetic pathway is illustrated in Scheme 8.¹⁴³ The synthetic route began with the reduction of dimethyl-5-hydroxyisophthalate (**3**) to yield the corresponding dialcohol (**4**). Subsequently, compound **4** was reacted with N-(4-bromobutyl)phthalimide to form the ether compound **5**. Through an Appel reaction, compound **5** was then converted into the dibromo derivative **6**. Compound **6** was further reacted with dipicolylamine to obtain compound **7**. Finally, hydrazinolysis of compound **7** led to the desired BDPA-Acid **8**. The intermediates and the final product (**8**) were characterized using ¹H NMR spectroscopy.

3.4.6 Synthesis of the BDPA-Acid (**12**) Ligand



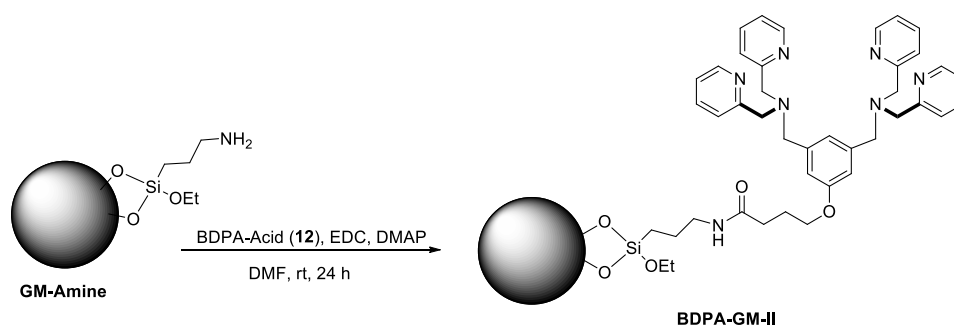
Scheme 10: Preparation of BDPA-Acid (**12**).

BDPA-Acid ligand **12** is suitable for conjugation to GM-Amine. The synthesis of ligand **12** was carried out based on a previous literature report, and the synthetic

pathway is summarized in Scheme 10.¹⁴⁴ The synthetic route begins with the reduction of dimethyl-5-hydroxyisophthalate (**3**) to obtain the corresponding dialcohol (**4**). Subsequently, compound **4** was reacted with ethyl 4-bromobutyrate to form the ether compound **9**. Through an Appel reaction, compound **9** was then converted into the dibromo derivative **10**. Compound **10** was further reacted with dipicolylamine to yield the BDPA-ester **11**. By subjecting compound **5** to basic hydrolysis, followed by careful work-up, the desired BDPA-acid **12** was obtained. The identities of all intermediates and the final product, **12**, were confirmed through ¹H NMR spectroscopy.

3.4.7 BDPA Derivatized Glass Microspheres

Derivatization GM-Amine with BDPA-Acid (**12**) to give BDPA-GM-II was attempted as summarized in Scheme 9. After the reaction, ninhydrin test was negative and ATR spectra were taken for the starting material and product. Three peaks were observed at 1640 cm⁻¹, 1547 cm⁻¹, and 1445 cm⁻¹ which indicate the presence of C=O (amide), N-H, and C-N bonds, respectively (Figure 36).¹⁴⁵ These new GMs were, however, to bind bacterial cells. Therefore, it could be concluded that BDPA-GM-II was obtained but the yield was too low to give GMs capable of binding sufficient numbers of bacterial cells. This derivatization route will be optimized in future work.



Scheme 9: Preparation of BDPA-GM-II.

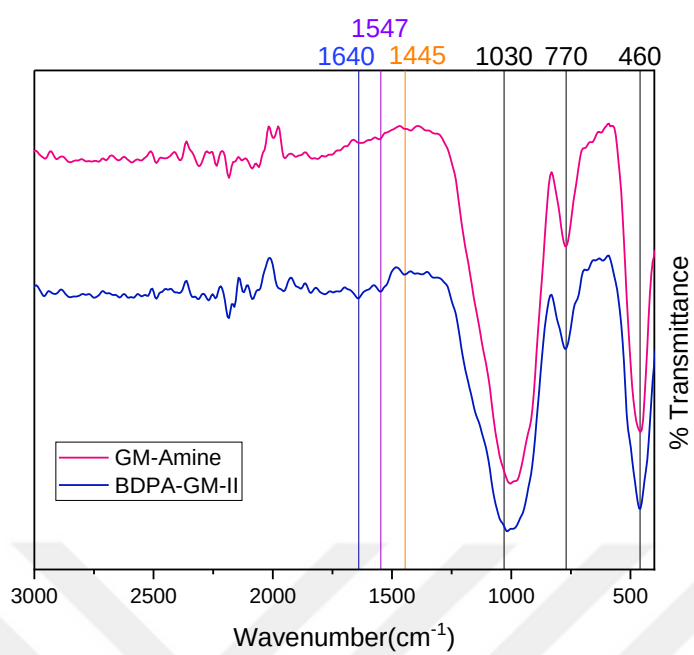


Figure 36. ATR-FTIR spectra of GM-Amine and BDPA-GM-II.

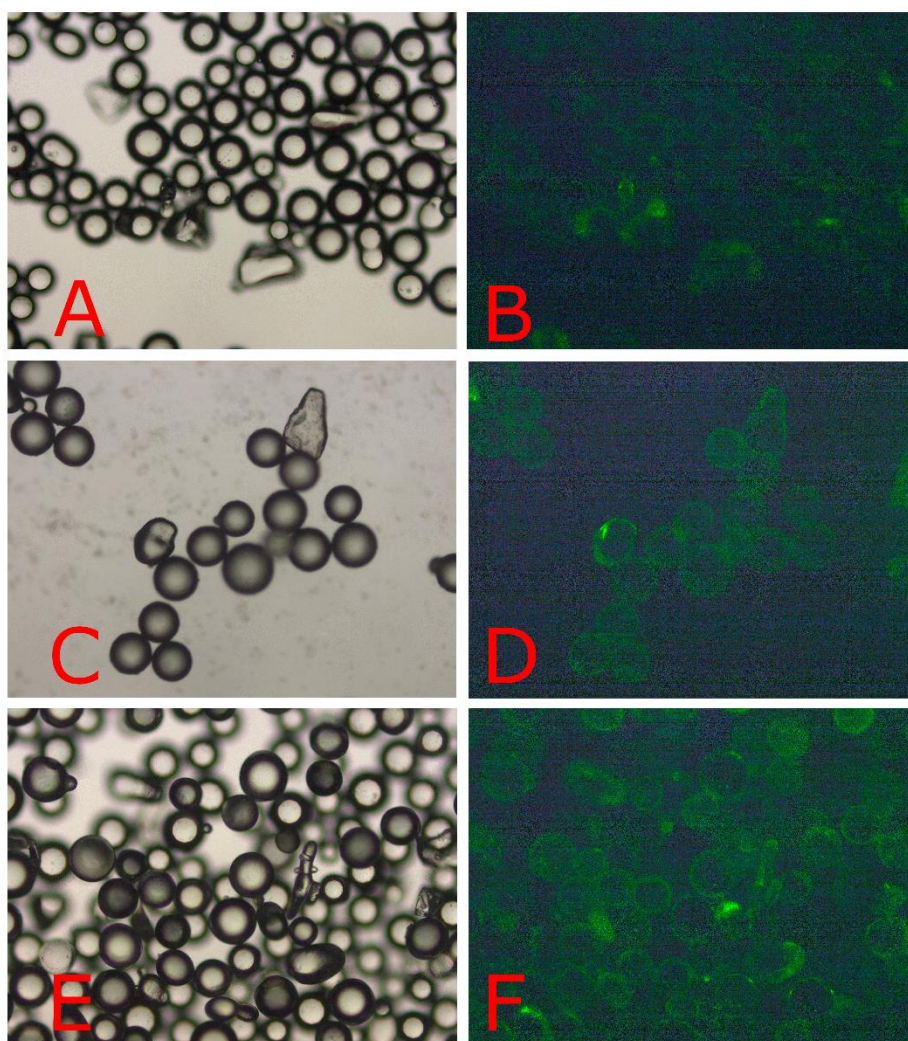


Figure 37. Bright field micrographs (10x) of GM **A**, GM-Amine **C**, BDPA-GM-II **E**, and fluorescence micrographs (10x, GFP filter) of GM **B**, GM-Amine **D**, BDPA-GM-II **F** after treatment with GFP-expressing *E. coli* K12 and washing.

The investigation of the affinity between bacterial cells and BDPA-GM-II was examined by investigating the interaction between GFP-expressing *Escherichia coli* (K12 pSB1C3-GFP) and various functional GMs was studied using fluorescence microscopy. The experiment involved mixing 100 mg aliquots of the three functional CMs with 250 μ L dispersions of *E. coli* cells at a bacterial cell number of 10^6 CFU/mL. The mixtures were then incubated, washed, and examined under fluorescence microscopy using a GFP filter. Figure 37 shows brightfield and fluorescence micrographs comparing BDPA-GM-II with its control GM-Amine and

GM. It is evident that GM-Amine exhibit some level of affinity towards bacterial cells. However, the difference between GM-Amine and BDPA-GM-II shows that BDPA ligand increases the affinity towards bacterial cells. However, the difference is not in expected levels. Therefore, optimization of GM derivatization is required.



CHAPTER 4

CONCLUSION

The objectives of these studies were to develop an aPDT system with Zn₂BDPA-bearing porphyrin derivative and liposomal encapsulation of Zn₂BDPA-bearing porphyrins to improve aPDT efficiency and, finally, to have Zn₂BDPA-bearing glass microspheres to sequester bacteria from aqueous liquid media.

In this study, the antimicrobial photodynamic activity of Zn₂BDPA-bearing porphyrin systems was investigated. Initially, the experimental procedure involved colony counting, with the bacterial suspension containing an excess of Zn(NO₃)₂. However, it was discovered that the excess Zn(NO₃)₂ had a toxic effect on bacterial cells. Subsequently, the toxicity of Zn(NO₃)₂ was examined, revealing that concentrations above 1 µM were toxic to bacterial cells. The minimum inhibitory concentration (MIC) of Zn(NO₃)₂ on *S. aureus* was determined to be 1.25 mM, with a minimum bactericidal concentration (MBC) of 10 mM. Although concentrations below 1.25 mM exhibited a bactericidal effect, caution should be exercised when using Zn(NO₃)₂ in experiments. Following the discovery of Zn(II) toxicity, the experiments were repeated. The bacterial cell number was reduced to 1x10⁶ CFU/mL, and the addition of excess zinc ions was omitted. The light and dark toxicity of Zn₂BDPA-I-Porphyrin **1d** at concentrations ranging from 1-20 µM were investigated against the Gram (+) bacterial species *S. aureus* and *B. cereus* and the Gram (-) bacterial species *E. coli* and *P. aeruginosa*.

Complete photoinactivation, characterized by a reduction of at least a log₆ in the number of viable bacterial cells, was observed for the Gram (+) bacterial species *S. aureus* and *B. cereus* at a 5 µM concentration of **1d**. Complete photoinactivation at the same concentration was also observed for the Gram (-) bacterial species *E. coli*

but not for the Gram (-) *P. aeruginosa*. Dark toxicity was also observed, and the reason behind dark toxicity was investigated. To investigate the structural origin of the dark toxicity, different fragments of **1d** and a similar derivative without the iodine atoms (**1a**) were selected, and their bacterial inactivation effects were investigated. It is understood that fragments were not the reason behind the dark toxicity, and membrane disruption can be the reason since the binding of **1a** to the membranes bacterial cells, but no passage into the cells, was shown through confocal microscopy.

Subsequently, a new procedure was introduced, namely broth microdilution, to get quicker results. To practice and ensure the reproducibility of the procedure, common antibiotics, namely ciprofloxacin, rifampicin, amoxicillin, and vancomycin, were tested on bacterial strains, including *E. coli* ATCC 25922, *S. aureus* ATCC 25923, *B. cereus* ATCC 14579, *P. aeruginosa* ATCC 9027, *P. aeruginosa* ATCC 27853, and *B. subtilis* ATCC 6633. The MIC values of the antibiotics were found to be consistent with the values reported in the literature.

Another objective was to achieve liposomal encapsulation of Zn₂BDPA-bearing porphyrin, **2**. Three different liposomal formulations were developed to encapsulate molecule **2**. Although successful encapsulation was achieved, it did not result in improved bacterial inactivation compared to the experiments with free **2**. As part of future work, new liposomal encapsulation procedures will be explored.

Studies towards Zn₂BDPA-bearing glass microspheres were also carried out with the ultimate objective of obtaining new materials for the sequestration of bacterial cells from aqueous liquid media such as blood. To this end, the glass microspheres were first derivatized to bear primary amine groups, GM-Amine, followed by the addition of the terminal carboxylic acid group, GM-Acid. The Kaiser test confirmed the successful formation of GM-Amine, and the IR data indicated the successful synthesis of GM-Acid and BDPA-GM-II. However, the obtained yields were not as high as expected, indicating the need for further optimization. Once the yields have been improved, the next step will involve testing the ability of the glass microspheres

to sequester bacteria from aqueous liquid media. If the glass microspheres can be successfully derivatized with high yields and possess a significant amount of BDPA groups, they hold the potential to effectively capture bacteria from liquid media, including blood. The need for time-consuming culture-based methods or complex sample preparation procedures can be reduced by directly capturing bacteria from liquid media. This not only saves valuable time in diagnosing bacterial infections but also provides a more reliable and rapid response, crucial in critical situations where immediate treatment is required.





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APPENDICES

A. NMR Spectra

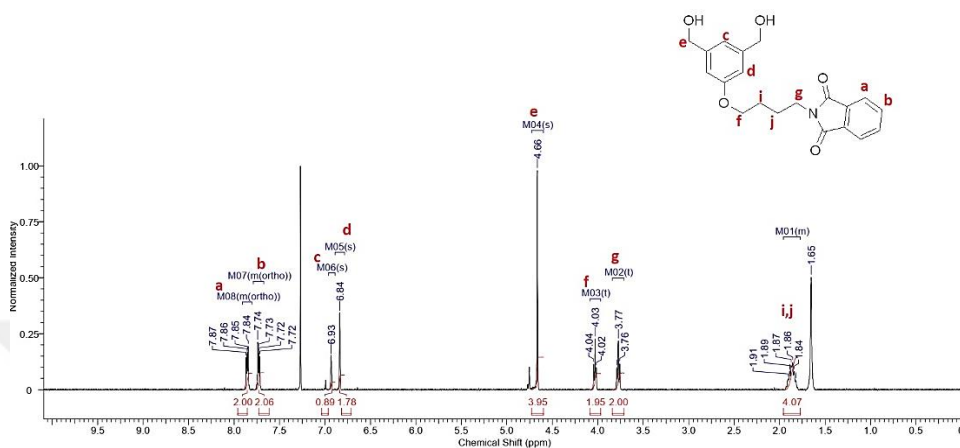


Figure A. 1. NMR spectrum of compound 5.

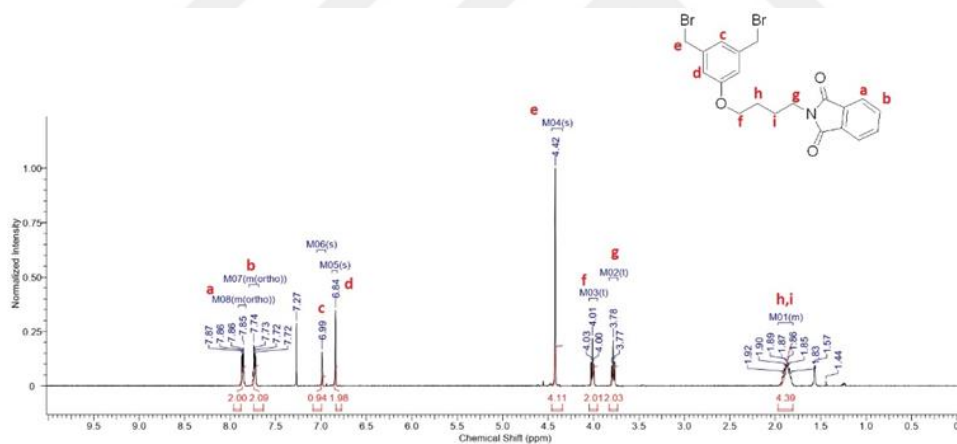


Figure A. 2. NMR spectrum of compound 6.

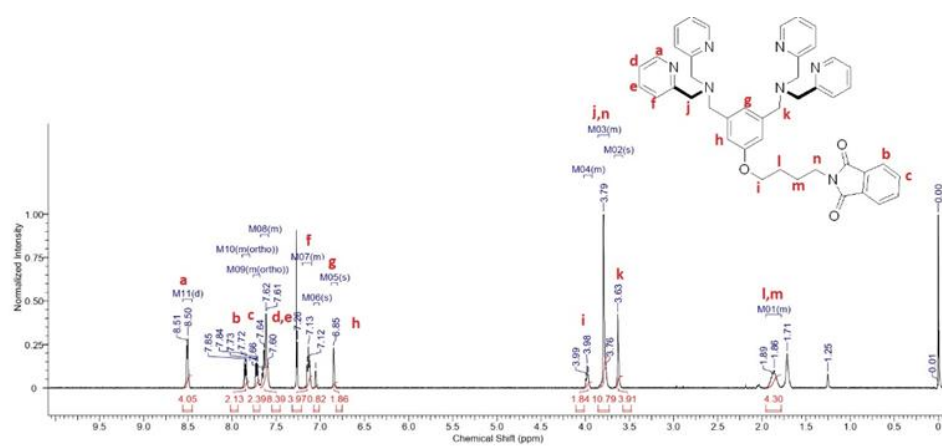


Figure A. 3. NMR spectrum of compound 7.

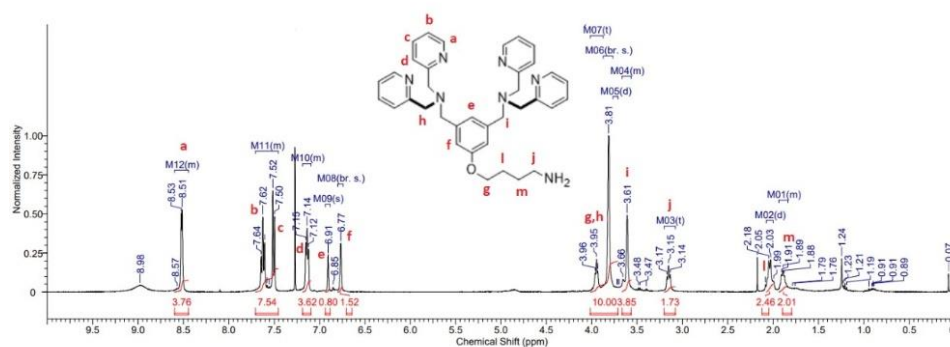


Figure A. 4. NMR spectrum of compound BDPA-Amine 8.

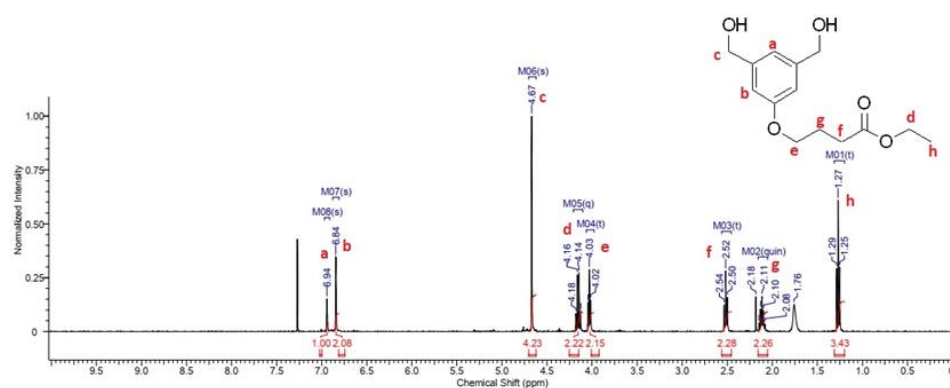


Figure A. 5. NMR spectrum of compound 9.

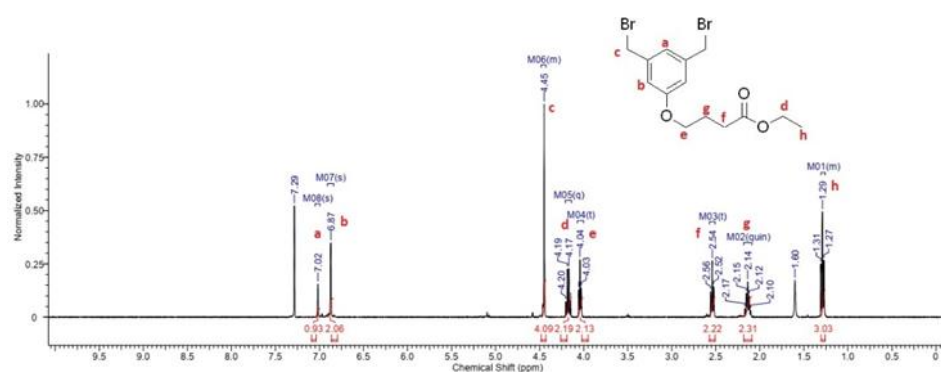


Figure A. 6. NMR spectrum of compound 10

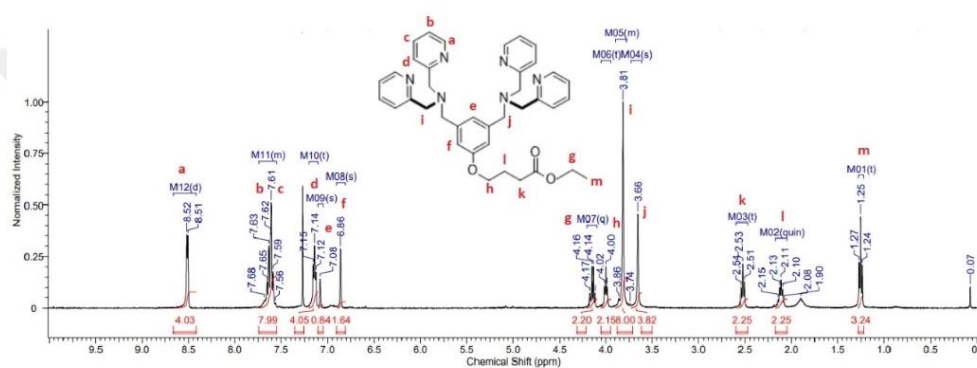


Figure A. 7. NMR spectrum of compound 11.

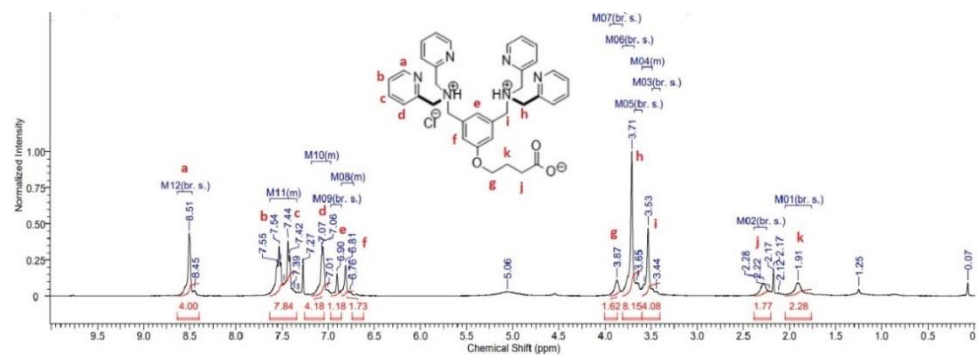


Figure A. 8. NMR spectrum of compound BDPA-Acid 12.

B. Dark Control Experiments for aPDT of 1d

Table A 1. Colony number on dark control plates for bacterial aPDT experiments of **1d**. Each experiment was repeated at least three times independently in three replicates.

Bacterial Strain	Dark Controls (expected 500 colonies)
<i>P. aeruginosa</i> ATCC 9027	500 ± 94
<i>S. aureus</i> ATCC 25923	454 ± 146
<i>E. coli</i> Top10	546 ± 151
<i>B. cereus</i> ATCC 14579	459 ± 51

Table A 2. OD₆₀₀ values for each strain.

Bacterial Strain	OD ₆₀₀
<i>P. aeruginosa</i> ATCC 9027	0.32= 10 ⁸ CFU/mL
<i>S. aureus</i> ATCC 25923	0.60= 5x10 ⁸ CFU/mL
<i>E. coli</i> Top10	0.25= 10 ⁸ CFU/mL
<i>E. coli</i> ATCC 25922	0.18= 10 ⁸ CFU/mL
<i>B. subtilis</i> ATCC 6633	2.8= 10 ⁸ CFU/mL
<i>B. cereus</i> ATCC 14579	0.65= 10 ⁸ CFU/mL
<i>P. aeruginosa</i> ATCC 27853	0.32= 10 ⁸ CFU/mL