ABSTRACT

CARPENTER, BRADLEY LYNN. From Angstroms to Millimeters: the Scalability of Antimicrobial Photodynamic Inactivation. (Under the direction of Dr. Reza A. Ghiladi).

The healthcare system is currently facing a serious threat caused by the rise of antibiotic-resistance in bacteria and yeast. These microbes are currently out maneuvering our chemotherapeutic development, and if left unchecked this has the potential to lead to a ‘post-antibiotic era’. Due to this reality, alternative strategies for the treatment and prevention of microbial infections are needed. Antimicrobial photodynamic inactivation (aPDI), which employs a photosensitizer, visible light, and molecular oxygen to inactivate microorganisms through the generation of cytotoxic singlet oxygen, is one such example of an alternative that shows great promise for the treatment of infections without leading to drug-resistance. However, to determine the technical limitations and the potential for aPDI to assist in the ongoing challenge of treating microbial infections, the further explorations of novel photosensitizers, targeting strategies, and pharmaceutical considerations are necessary.

Towards this end, Chapter 2 describes the investigation of a BODIPY photosensitizer for aPDI. Currently, very few studies of BODIPY-based photosensitizers have been reported, yet there is a growing interest in this class of compounds for aPDI due to their small size, ease of synthetic modification, and tunable absorption features. To investigate this scaffold, 2,6-diiodo-1,3,5,7-tetramethyl-8-(N-methyl-4-pyridyl)-4,4’-difluoroboradiazaindacene (DIMPy-BODIPY) for its antibacterial, antifungal, and antiviral activities. The results demonstrated that DIMPy-BODIPY was capable of the photoinactivation of bacteria, fungi, and viruses with outstanding efficacy, suggesting the BODIPY scaffold is an effective photosensitizer for the development of broadly applicable anti-infective agents.

The majority of nosocomial infections are caused by the transmission of pathogens from surfaces or materials to patients in hospital environments. Another strategy to reduce deaths associated with nosocomial infections is the use of advanced technologies and materials for hospital sterilization. While aPDI has predominately been viewed as a means of treating infections, antimicrobial materials which use a photodynamic mode of action have great
promise to also assist in surface and material sterilization. However, studies are needed to determine the efficient means for the preparation of such materials, to characterize their physical properties, and to ascertain what characteristics result in the greatest antimicrobial efficacy. In Chapter 3, we report the preparation and antimicrobial properties of a cationic photosensitizer covalently attached to cellulose nanocrystals. Termed CNC-Por (1), this nanoscale material was investigated for its ability to photoinactivate taxonomically diverse bacteria, and was found to be a highly effective antibacterial agent. Using confocal fluorescence microscopy, it was determined that photosensitizer internalization was not a necessary prerequisite for antibacterial inactivation, confirming a material-based mechanism of inactivation rather than one based upon a soluble photosensitizer.

In Chapter 4, a diverse set of photosensitizers were conjugated to cellulose fibers and subsequently pressed into paper sheets, yielding photomicrobicidal papers. The physical properties and antimicrobial efficacy of these materials were subsequently explored. A cationic porphyrin-modified paper, Por\(^{(+)}\)-paper, was found to be the most effective against bacteria, inactivating each strain studied by ~4 log units reduction in CFU/mL. Due to its efficacy against bacteria, we also explored this material for antiviral PDI, and observed detection limit inactivation of both dengue-1 virus (4.5 log units) and influenza A (2.5 log units). Human adenovirus-5 was found to be slightly more resistant to photoinactivation, but a notable 99% reduction in FFU/mL (2 log units) was still achieved. These results demonstrate the versatility of this conjugation strategy with diverse photosensitizer and cellulose substrates, and the potential of materials with the antimicrobial photodynamic inactivation mode of action to mediate the sterilization of hospital as well as other environments.
From Angstroms to Millimeters: the Scalability of Antimicrobial Photodynamic Inactivation
North Carolina State University

by
Bradley Lynn Carpenter

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APPROVED BY:

Dr. Jonathan Lindsey
Dr. Dimitris Argyropoulos

Dr. Elon Ison
Dr. Walter Weare

Dr. Reza Ghiladi
Chair of Advisory Committee
BIOGRAPHY

Brad Carpenter was born on August 14th 1987 in Dallas, Texas to Daniel and Janice Carpenter. While spending his formative years in Texas, he learned the values of good BBQ, Tex-Mex, and family. At the age of 9 he moved to Charlotte, North Carolina. He spent a great deal of his time playing outdoors and refused to wear shoes except when at school. Like many children in his generation, he became swept-up in the rollerblades movement. He also became an avid reader of historical fiction, science fiction, and classical literature. While attending Butler High school, Brad was challenged to begin to take his studies seriously, and with the development of some discipline (turning in the majority of assignments) was able to move from average grades (Bs and Cs) to above average (As and Bs), eventually finishing in the top 25 % of his competitive high school class. After graduating from high school, Brad entered Campbell University as a Pre-engineering major, with the expectation to transfer to an engineering school in two years. With effort, Brad achieved excellent grades and was accepted by all the engineering departments to which he had applied. However, due to his success in and enjoyment of Chemistry and Biology classes, Brad decided to remain at Campbell with a dual major in Biochemistry and Chemistry. After graduating Magna Cum Laude, he chose to pursue his interest in research by joining the North Carolina State University department of chemistry on a PhD track. With some bumps and indecision, he eventually joined the lab of Professor Reza A. Ghiladi, taking on the project described herein. After grappling with the challenges of finding order in chaos, he eventually (through the great efforts of his esteemed advisor) defended his PhD in 2015 and will be continuing his career in research at the Georgia Institute of Technology under the guidance of Professor Wendy Kelly. Though he was not born in North Carolina, Brad Carpenter will forever love the state of blue mountains, green rolling hills, and majestic waters. In short, he will remain a Tar Heel.
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Chapter 1

Introduction

1.1 A Brief History of Antimicrobial Photodynamic Inactivation

Photodynamic therapy, or PDT, is a treatment modality that relies on a dye, termed a photosensitizer, and light to selectively kill either cancerous tissues or pathogens. While PDT may seem like a modern high-tech therapeutic, it was actually conceived over 100 years ago, when Raab recognized the combination of a dye, acridine orange, and visible light was toxic to microbial organisms.\(^1\) It was later shown that oxygen was a requirement for mediating this toxicity and for this reason it was termed “photodynamic.”\(^2\) Little research was reported on PDT until a resurgence in the 1960s and 1970s, when it was envisioned as a treatment for cancer.\(^3\) Due to the effectiveness of antibiotics and some of the difficulties associated with implementing a new treatment modality, research into antimicrobial photodynamic inactivation (aPDI) did not dramatically increase until the last 20 years.\(^2\) Throughout the “antibiotic era,” new antibiotics were frequently thought to be the final cure for bacterial disease, shortly before drug resistant strains were discovered.\(^4,5\) It wasn’t until the early 1990s, however, that scientists came to understand that microbes have the potential to resist all new antibiotics, and with the added limitation of fewer new antibiotics being discovered, they termed the 1990s “the beginning of the post-antibiotic era.”\(^5\) It was at this point that aPDI once again became a viable area of research, as finding new methods for preventing and treating microbial infections without the development of resistance was realized to be a dire necessity.

1.2 The Mechanisms of PDT

There are two general routes to photosensitization, referred to as the type 1 and type 2 pathways of photosensitization. As seen in Figure 1.1, the photosensitizer is first excited from its ground electronic state to its first excited singlet state by the absorption of visible light (1). From there it can either follow a relaxation pathway back to its ground state (2) or go through
an intersystem crossing to reach its first triplet excited state (4). It is very important for a good photosensitizer to have rapid intersystem conversion and slow relaxation pathways. Once in the triplet state, the photosensitizer can either transfer its energy to molecular oxygen forming singlet oxygen, \(^1\text{O}_2\), or reacts to form radical species. These radical species are typically either superoxide or hydroxide radicals. Both singlet oxygen and radical species are very reactive and cytotoxic, so they are both considered photosensitization pathways. Radical species are considered the outcome to type I photosensitization, while singlet oxygen is the outcome to type II photosensitization. It has been demonstrated that most photosensitizers predominately operate through a type II pathway.\(^2\) Singlet oxygen has a short lifetime, and when its rate of diffusion in cells is factored in it has a 155 nm “sphere of activity,” meaning that singlet oxygen only reacts with and damages cellular structures within 155 nm of the photosensitizer where it is excited.\(^6\)
Figure 1.1. Jablonski diagram showing type I and type II photosensitization (adapted from reference 7). (1) Represents the absorption of light, exciting the photosensitizer to an excited singlet state followed by relaxation to the first excited singlet state. (2) Represents relaxation by fluorescence. (3) Represents non-radiative relaxation. (4) Represents intersystem crossing and relaxation to the first excited triplet state. (5) Represents relaxation by phosphorescence. (6) Represents non-radiative relaxation. (7) Represents redox reactions forming radicals. (8) Represents type I photosensitization. (9) Represents the excitation of ground state molecular oxygen to its singlet state, and subsequent relaxation to its first excited singlet state. (10) Represents type II photosensitization.

1.3 Antimicrobial Photodynamic Inactivation

To treat any infectious disease there must be selectivity for the drug to attack the pathogen and not the host tissue. The classic example of this selectivity comes from penicillin, which attacks bacterial cell wall synthesis. Since mammalian cells don’t have cell walls, this drug does not interfere with their life cycle, while for bacteria the inability to produce a cell wall is devastating. One of the most promising aspects of aPDI is that it has several selectivities. Most photosensitizers are designed so as to localize on the surface of or be internalized into the target pathogen. There is a second selectivity in illumination, since the photosensitizer should be non-toxic when kept in the dark, cytotoxic species will be present only where the dye is illuminated. There is also the selectivity of cell size. Bacterial cells are about 2,000 times smaller than the mammalian cells they infect, and for this reason it is much
more likely that singlet oxygen in a bacterial cell will damage essential cellular structures than singlet oxygen in the mammalian cell. All of these selectivities combine to make aPDI a viable treatment option for infectious disease. However, while aPDI is a viable treatment modality, designing photosensitizers that best utilize these selectivities is an ever present challenge.

While some photosensitizer designs are aimed at specific pathogens, there are many general properties required of a good photosensitizer. A photosensitizer should have a large absorptivity in the phototherapeutic window, 620–850 nm, so that it can be efficiently excited through tissue. As mentioned previously, a photosensitizer must also efficiently undergo an intersystem crossing from its first excited singlet state, to its first excited triplet state in order to have a large singlet oxygen quantum yield. The photosensitizer should be non-toxic, to increase the selectivity of illumination. Finally, and perhaps most importantly, the photosensitizer must selectively localize either in the cellular envelope of the pathogen, and away from sensitive mammalian cellular structures.

1.4 Targeting Bacteria with Photosensitizers

It is this targeting of microbial pathogens that is the focus of much aPDI research. A great deal of effort has been invested into discovering the best ways to locate photosensitizers on the surface or internalized into bacteria. As stated previously, with the increasing prevalence of drug-resistant bacteria, new methods of treating bacterial infections are highly desirable. For aPDI to be considered a viable replacement for antibiotics, it needs to have broad spectrum antibacterial activity, and this has been the greatest design challenge for photosensitizers. Current research suggests that most effective antibacterial photosensitizers locate on the surface and not internalized into bacteria. Unfortunately, developing broad spectrum photosensitizers that efficiently locate on the bacterial surface is made extremely difficult by the diversity of bacteria.

Generally bacteria are broken down into two categories, Gram-positive and Gram-negative. The main difference in the two categories is the presence of an extra membrane, called the outer-membrane which is located on the external surface of Gram-negative bacteria.
beyond their cell wall (Figure 1.2). Gram-positive species do not have this membrane, so their external surface is comprised solely of a cell wall and a cytoplasmic membrane. So a broad spectrum photosensitizer must both interact well with the outer-membrane of Gram-negative bacteria and the cell wall/cytoplasmic membrane of Gram-positive bacteria. To add even more difficulty, there is much variation between species and even strains of bacteria in the same category, and many kinds of bacteria have unique external structures that do not fit into Gram categories at all. The most common targeting strategy used to overcome this difficulty is using cationic charges. Since both Gram-positive and Gram-negative have anionic surface charges, a cationic photosensitizer can develop an electrostatic interaction with the bacterial membranes of both. This strategy has proven so effective that nearly all new photosensitizers reported utilize cationic charges to some extent. However there are still many challenges left to overcome.
Figure 1.2. A comparison of Gram-positive and Gram-negative bacterial surfaces (adapted from reference 11).
1.5 Challenges in Antimicrobial Photodynamic Inactivation

There are still many bacterial strains that remain resistant to photodynamic inactivation, most of them being Gram-negative species. The means of these species’ and strains’ resistance to aPDI is unknown, though several phenotypic suggestions have been put forward. Studies into the bacterial phenotypes that allow resistance to inactivation are vital. Another challenge for bacterial infections in general is overcoming biofilms. Most drug-resistant infections rely on biofilm formation, yet currently there is very little literature on photodynamic inactivation of bacteria when in a biofilm. To truly replace antibiotics, aPDI requires much better broad-spectrum antibacterial activity, and this could require a new targeting modality beyond that of cationic charges.

Translational challenges also exist in the conversion from effective aPDI of pathogens in vitro, to effective in vivo treatment of infected patients. It has been shown that treating infections in vivo requires much higher concentrations of photosensitizer due to localization and illumination challenges. Another challenge lies in the added variable of illumination. Doctors are accustomed to simply coming up with a drug dosage, but aPDI is much more complicated. Doctors would be required to come up with a dosage, a light intensity, and a length of illumination. Simply put, it will take great results to convince doctors to change their prescription mentality. Currently there are very few FDA approved photosensitizers, and none of them are approved for the treatment of infections, though several pharmaceutical companies have new photosensitizers currently in clinical trials.

1.6 Conclusion

In order to stay ahead of the emerging drug-resistant microbial pathogens, great efforts to explore new treatment modalities will be required. One such treatment that shows great potential is antimicrobial photodynamic therapy. Though slightly more complicated than simply taking a pill, aPDI’s selectivity, as well as its ability to avoid bacterial drug-resistance makes it a viable treatment option. Much work however still needs to be done to improve its
efficacy against challenging bacterial strains that are resistant to current photosensitizers, and to simply treatment procedures.

1.7 References


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Chapter 2
Antiviral, Antifungal and Antibacterial Activities of a BODIPY-based Photosensitizer

Bradley L. Carpenter,† Xingci Situ,† Frank Scholle,‡ Juergen Bartelmess,† Walter W. Weare,† and Reza A. Ghiladi†*

† Department of Chemistry, North Carolina State University, Raleigh, NC 27695-8204; E-Mails: blcarpen@ncsu.edu (B.L.C.); xsitu@ncsu.edu (X.S.); jbartel@ncsu.edu (J.B.); wwwearwe@ncsu.edu (W.W.W.)
‡ Department of Biological Sciences, North Carolina State University, Raleigh, NC 27695-7614; E-Mail: fscholl@ncsu.edu (F.S.)
* Author to whom correspondence should be addressed; E-Mail: Reza_Ghiladi@ncsu.edu; Tel.: +1-919-389-1716; Fax: +1-919-515-5079.

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2.1 Abstract: Antimicrobial photodynamic inactivation (aPDI) employing the BODIPY-based photosensitizer 2,6-diiodo-1,3,5,7-tetramethyl-8-(N-methyl-4-pyridyl)-4,4’-difluoroboradiazaindacene (DIMPy-BODIPY) was explored in an in vitro assay against six species of bacteria (8 total strains), three species of yeast, and three viruses as a complementary approach to their current drug-based or non-existent treatments. Our best results achieved a noteworthy 5–6 log unit reduction in CFU at 100 nM for Staphylococcus aureus (ATCC-2913), methicillin-resistant S. aureus (ATCC-44), and vancomycin-resistant Enterococcus
faecium (ATCC-2320), a 4-5 log unit reduction for Acinetobacter baumannii ATCC-19606 (250 nM), multidrug resistant A. baumannii ATCC-1605 (100 nM), Pseudomonas aeruginosa ATCC-97 (500 nM), and Klebsiella pneumoniae ATCC-2146 (1 μM), and a 3 log unit reduction for Mycobacterium smegmatis mc²155 (ATCC-700084). A 5 log unit reduction in CFU was observed for Candida albicans ATCC-90028 (1 μM) and Cryptococcus neoformans ATCC-64538 (500 nM), and a 3 log unit reduction was noted for Candida glabrata ATCC-15545 (1 μM). Infectivity was reduced by 6 log units in dengue 1 (100 nM), by 5 log units (500 nM) in vesicular stomatitis virus, and by 2 log units (5 μM) in human adenovirus-5. Overall, the results demonstrate that DIMPy-BODIPY exhibits antiviral, antibacterial and antifungal photodynamic inactivation at nanomolar concentrations and short illumination times.

**Keywords:** photodynamic therapy; singlet oxygen; antibacterial; antiviral; antifungal; photobiocidal

### 2.2 Introduction

Despite the advent of antibiotics and vaccines, infectious diseases remain the leading cause of mortality and morbidity worldwide [1]. Accounting for over 60% of deaths in the developing world, they are also the third and fourth leading causes of death in Europe and the United States, respectively [1,2]. Efforts to control microbial infections have been hampered by the emergence and proliferation of drug resistant pathogens, necessitating the pursuit of complementary approaches to the current drug-based treatments. Furthermore, a scarcity of effective therapies for many globally important viral infections stresses the need for development of novel approaches for either treatment or prevention of infection. One such option, antimicrobial photodynamic inactivation (aPDI), is currently being explored as a potential therapeutic treatment option for various types of infection, whether bacterial, fungal, viral, or even parasitic in nature [3-7]. aPDI makes use of a photosensitizer (PS) to generate reactive oxygen species (i.e., radicals or singlet oxygen ($^{1}\text{O}_2$)) upon illumination with light
Although challenges with aPDI exist, such as issues of tissue penetration with light and photosensitizer selectivity, as a biocidal agent, $^1$O$_2$ possesses a number of unique properties that make it particularly attractive for antimicrobial applications. These include damaging reactivity with most biomolecules, a short lifetime of $\sim 10^{-6}$ s in aqueous environments, and the formation of harmless ground state molecular oxygen if left unreacted [8,9]. More importantly, development of bacterial resistance to aPDI is believed to be unlikely due to the non-specific damage caused by $^1$O$_2$ [10]. Additionally, a number of studies have shown that a photodynamic inactivation strategy is equally effective against both drug-susceptible and drug-resistant bacterial strains, demonstrating the enormous potential of aPDI in combating pathogenic infections [11,12].

While numerous efforts have focused on photosensitizer scaffolds based upon cationic tetapyrrole-related macrocycles (porphyrins, bacteriochlorins, phthalocyanines) or other conjugated systems [13-16], in particular methylene blue [17-20], very little is known regarding the applicability of boron dipyrromethene (a.k.a. BODIPY)-based compounds as potential photosensitizers for aPDI. Of particular interest for our work are two recent studies: the first, by Caruso et al. [21], investigated two novel cationic and iodinated BODIPYs as photosensitizers, but only against two bacterial model strains, *Escherichia coli* and *Staphylococcus xylosus*. The second study, by O'Shea et al. [22], examined the aPDI application of the structurally-related aza-BODIPY photosensitizers against *E. coli*, *S. aureus*, and *Candida albicans*. Outside of these two studies, reports of a BODIPY-based photosensitizer employed in antimicrobial photodynamic inactivation are limited, and to the best of our knowledge no studies have been performed against viruses with this class of photosensitizer. Given that the majority of nosocomial infections in the United States are caused by *Staphylococcus aureus* (15%), enterococci species (12%), candida species (11%), *Pseudomonas aeruginosa* (8%), *Klebsiella pneumoniae* (6%), enterobacter species (5%) and *Acinetobacter baumannii* (3%) [23,24], the fact that relatively few clinically-relevant pathogenic bacterial and fungal species, and importantly no viruses, have been explored with BODIPY-based systems highlights a lack of understanding of this new class of photosensitizer.
with pathogens of direct importance to human health and disease. Herein, to further investigations into the feasibility of aPDI for future therapeutic use as a complementary approach to drug-based treatments, we explored the extent of *in vitro* antimicrobial photodynamic inactivation employing the DIMPy-BODIPY [21,25] photosensitizer (Figure 2.1) against eight species of bacteria and three species of yeast that together fall within the five classes of antibiotic-resistant pathogens that are emerging as major public health threats: vancomycin-resistant enterococci (VRE), methicillin-resistant *Staphylococcus aureus* (MRSA), multidrug-resistant mycobacteria, Gram-negative bacteria, and fungi [26]. We also extended our study to include dengue-1 virus, vesicular stomatitis virus (VSV), and human adenovirus-5 as model DNA viruses to explore the potential of antiviral PDI [27-30] using BODIPY-based photosensitizers for non-*in vivo* surface sterilization or materials applications.

![DIMPy-BODIPY photosensitizer](image)

**Figure 2.1.** The DIMPy-BODIPY photosensitizer employed in this study.

### 2.3 Results and Discussion

#### 2.3.1 Antiviral photodynamic inactivation studies

The potential efficacy of DIMPy-BODIPY as a photodynamic inactivator of viral agents was tested using three viruses from different families: the enveloped viruses dengue virus type I (*Flaviviridae*) and vesicular stomatitis virus (*Paramyxoviridae*), and the non-
enveloped virus human adenovirus-5 (*Adenoviridae*). Viruses were incubated with different concentrations of DIMPy-BODIPY and illuminated (400-700 nm, 65±5 mW/cm²) as described or kept in the dark as a negative control. Virus infectivity after treatment was determined by plaque (VSV, HAd) or immunofocus assay (dengue) on Vero cells (Figure 2.2). In the absence of illumination, the infectivities of dengue and VSV were not affected at any of the concentrations of photosensitizer tested (Figure 2.2A). In contrast, after illumination, dengue virus was inactivated below the level of detection at DIMPy-BODIPY concentrations of 1 μM and 0.1 μM, which represents a drop in infectivity of over 6 log units reduction in FFU/mL (*P*<0.001). Even at the low concentration of 10 nM DIMPy-BODIPY, dengue was partially inactivated, with the infectivity showing a reduction of approximately 2 log units in FFU/mL (*P*<0.001).
Figure 2.2. Photodynamic inactivation as a function of DIMPy-BODIPY concentration of (A) dengue 1 virus (blue) and vesicular stomatitis virus (VSV, red), and (B) human adenovirus-5 (HAd-5). Solid lines represent the light treated samples, whereas the dashed lines represent the dark controls. For these studies, the illumination conditions were as follows: 30 min, 400-700 nm, 65±5 mW/cm² (total fluence of 118 J/cm²). The detection limits of the plaque assays were 50 PFU/mL for the dengue virus study, 40 FFU/mL for the VSV study, and 66 PFU/mL for the HAd-5 study; data points below the detection limit were set to these values (represented by the shaded area) for graphing purposes. In the cases where error bars cannot be visualized, the error bars themselves were smaller than the marker employed in the plot.

Pilot experiments demonstrated that VSV proved to be slightly more resistant to inactivation than dengue at 0.1 µM DIMPy-BODIPY, remaining almost fully infectious (unpublished data). Therefore, for VSV the concentrations of DIMPy-BODIPY were varied from 0.25 – 1 µM. As with dengue, the infectivity of VSV was not negatively affected at any
of the DIMPy-BODIPY concentrations tested in the absence of illumination. After light treatment, however, VSV was completely inactivated at 1 μM DIMPy-BODIPY, representing a drop in infectivity of over 6 log units reduction in PFU/mL ($P<0.001$; Figure 2.2A). At 500 nM DIMPy-BODIPY, the viral infectivity was reduced nearly 5 log units in PFU/mL ($P<0.001$), and was similarly reduced by nearly 4 log units in PFU/mL ($P<0.001$) at the lowest effective concentration tested of 250 nM DIMPy-BODIPY.

In contrast to efficient photoinactivation of the enveloped viruses above, the non-enveloped human adenovirus-5 proved more resistant to inactivation by DIMPy-BODIPY. At 1 μM, a 1 log unit reduction in PFU/mL ($P<0.025$) was observed that increased to 2 log units ($P<0.001$) at a photosensitizer concentration of 5 μM. A possible explanation to the greater resistance of adenovirus to photoinactivation is that the virus capsid in non-enveloped viruses, which in HAd-5 is comprised of at least nine different proteins (32), affords a greater level of protection to the virion from its surrounding environment than the lipid membrane and associated proteins of enveloped ones. Interestingly, the dark control samples themselves showed ~80% reduction in PFU/mL for 1-5 μM DIMPy-BODIPY, suggesting a non-photoinduced virus inactivation pathway may also be present, but this was not further explored.

2.3.2 Antibacterial photodynamic inactivation studies

In vitro aPDI studies employing the photosensitizer DIMPy-BODIPY were performed in a concentration-dependent manner. The illumination time was fixed at 30 minutes for the bacterial studies as determined by a time-dependence study of the photobleaching of DIMPy-BODIPY (see Supplementary Data and Figure 2.S1). For the three Gram-positive bacteria *S. aureus* ATCC-2913, methicillin-resistant *S. aureus* (MRSA) strain ATCC-44, and the vancomycin-resistant *E. faecium* (VRE) strain ATCC-2320, they were found to be highly susceptible to photodynamic inactivation with DIMPy-BODIPY regardless of their respective antibiotic resistance (Figure 2.3A). For example, at the concentration of 100 nM, DIMPy-BODIPY reduced bacterial survival by 5-6 log units (99.999% viable cell eradication,
For all three after 30 min illumination (400-700 nm, 65±5 mW/cm²). At concentrations above 250 nM under the same illumination conditions, no surviving bacteria were detected for any of the three bacteria. By comparison, the benchmark photosensitizers TMPyP (Figure 2.S3A) and methylene blue (Figure 2.S4A) showed no statistically significant cell inactivation at 100 nM photosensitizer concentration, and were only able to achieve a 5 log units reduction in bacteria when 5- to 10-fold higher concentrations of the photosensitizers were employed (typically 1 μM or higher). Decreasing the DIMPy-BODIPY concentration to 50 nM led to a partial attenuation of the inactivation efficacy for both *S. aureus* ATCC-2913 (2.5 log units reduction in CFU/mL, *P*<0.001) and *E. faecium* ATCC-2320 (3.5 log units reduction, *P*<0.001), whereas there was no statistical difference observed for the MRSA strain ATCC-44 between 50 and 100 nM concentrations (both ~5 log units reduction). When the concentration of the photosensitizer was further decreased to 25 nM, each of the *S. aureus* strains ceased to be inactivated, while the *E. faecium* strain remarkably still exhibited 99% inactivation (2 log units, *P*<0.001). No statistically significant inactivation was seen at a DIMPy-BODIPY concentration of 10 nM for any of the three aforementioned bacteria.

The taxonomically Gram-positive bacterium *M. smegmatis* strain ATCC-700084, shown separately in Figure 2.4, exhibited ~4 log units inactivation at 1 μM (*P*<0.001), ~3.3 log units at 500 nM (*P*<0.001), and 95% inactivation (~2 log units, *P*<0.001) at 250 nM. No statistically significant inactivation of this *M. smegmatis* strain was observed at DIMPy-BODIPY concentrations below 100 nM. By comparison, methylene blue is a much poorer photosensitizer under identical illumination conditions, having previously been shown to photoinactivate *M. smegmatis* by 4 log units at only a very high concentration of 75 μM, by 2 log units at 7.5 μM, and showed no inactivation at 750 nM [13]. However, under the same conditions, TMPyP has been shown to be highly efficient in its photoinactivation of *M. smegmatis*, achieving detection limit inactivation (6+ log units reduction) at concentrations as low as 150 nM [13].
Figure 2.3. Photodynamic inactivation of bacteria as a function of DIMPy-BODIPY concentration. (A) Gram positive species. Displayed is the % survival of the dark control (♦) and the light treated samples for methicillin-susceptible *S. aureus* (MSSA) ATCC-2913 (■), methicillin-resistant *S. aureus* (MRSA) ATCC-44 (▲), and the vancomycin-resistant *Enterococcus faecium* (VRE) ATCC-2320 strain (xBC). (B) Gram negative species. Displayed is the % survival of the dark control (♦) and the light treated samples for *A. baumannii* ATCC-19606 (■), multidrug-resistant *A. baumannii* (MDRAB) ATCC-1605 (▲), *P. aeruginosa* ATCC-97 (xBC), and *K. pneumoniae* ATCC-2146 (♦). For all bacteria, the illumination conditions were as follows: 30 min, 400-700 nm, 65±5 mW/cm² (total fluence of 118 J/cm²). As the plating technique employed to determine % survival did not allow for detection of survival rates of <0.0001%, data points below the detection limit were set to 0.0001% survival for graphing purposes. The shaded areas correspond to undetectable cell survival with the assay employed. In the cases where error bars cannot be visualized, the error bars themselves were smaller than the marker employed in the plot.
Figure 2.4. Photodynamic inactivation of *Mycobacterium smegmatis* mc²155 ATCC-700084 as a function of DIMPy-BODIPY concentration. Displayed is the % survival of the dark control (♦) and the light treated samples (■). Illumination and assay conditions were as in Figure 2.3.

While the Gram-positive species were fairly consistent with respect to their photoinactivation when compared to one another, the Gram-negative ones (drug-susceptible *A. baumannii* strain ATCC-19606, the multi-drug resistant *A. baumannii* (MDRAB) strain ATCC-1605, *P. aeruginosa* strain ATCC-97 and *K. pneumoniae* strain ATCC-2146) had a wider range of susceptibilities to BODIPY-mediated aPDI (Figure 2.3B). When the efficacy of DIMPy-BODIPY at a concentration of 500 nM was examined against the two *A. baumannii* strains, no CFUs were detected, and survival rates were therefore below the detection limit of <0.0001%, corresponding to an impressive 6 log units reduction in viable cells (*P*<0.001). At 100 nM, DIMPy-BODIPY was still able to achieve a noteworthy 3 and 4.5 log units reduction in CFU for *A. baumannii* ATCC-19606 and MDRAB ATCC-1605, respectively, whereas 50 nM was the lowest PS concentration for which aPDI was statistically significant (57% and 77% cell eradication for *A. baumannii* ATCC-19606 and MDRAB ATCC-1605, respectively, *P*<0.02). No statistically significant inactivation of either strain was seen at a DIMPy-BODIPY concentration of 25 nM or lower. Thus, with the exceptions at the extremes of the
concentrations studied (above 500 nM or below 25 nM), the MDRAB ATCC-1605 strain was statistically more susceptible ($P = ~0.02$ or lower), albeit only slightly, to photodynamic inactivation by DIMPy-BODIPY when compared with A. baumannii ATCC-19606. By comparison, the commercial photosensitizers were poorer performers: TMPyP (Figure 2.S3B) required a 5-fold higher concentration (2.5 μM) to achieve 5 log units of inactivation, and methylene blue (Figure 2.S4B) required a 2-fold higher concentration (1.0 μM), with both exhibiting no statistically significant photoinactivation at or below 250 nM.

The P. aeruginosa ATCC-97 strain was also found to be inactivated by DIMPy-BODIPY at mid nM to low μM concentrations (Figure 2.3B): ~5.5 log units inactivation was observed ($P<0.001$) at a photosensitizer concentration of 1 μM. Lowering the concentration to the nM regime decreased the inactivation efficacy; however, it was still possible to achieve a notable ~4.5 log units reduction in CFU/mL at 500 nM ($P<0.001$), and 98% inactivation (~2 log units, $P<0.001$) at 250 nM. No statistically significant inactivation of this P. aeruginosa strain was observed at DIMPy-BODIPY concentrations below 100 nM. As a benchmark, methylene blue was able to match the highest observed level of photoinactivation (~5.5 log units; Figure 2.S4B), but required a 5-fold higher photosensitizer concentration (5 μM) to do so, whereas TMPyP (Figure 2.S3B) was only able to achieve 3 log units reduction in viable cells at that same concentration.

In comparison to the other bacterial species investigated, the multi-drug resistant NDM-1-producing K. pneumoniae clinical isolate ATCC-2146 explored here was the least susceptible to photodynamic inactivation by DIMPy-BODIPY, with no inactivation observed at 500 nM (Figure 2.3B). At 1 μM, a concentration that achieved a detection-limit level of inactivation for nearly all other bacteria examined, a 99.9% (3 log units) reduction in CFU/mL was obtained for this K. pneumoniae strain ($P<0.001$). In an attempt to see if near detection limit inactivation was possible, the photosensitizer concentration was increased to 2.5 μM, which showed ~4 log units of inactivation (>99.99%, $P<0.001$). By comparison, TMPyP was slightly more effective than DIMPy-BODIPY, reaching ~5 log units reduction at 2.5 μM.
(Figure 2.S3B), whereas methylene blue was a far poorer photosensitizer, exhibiting no statistically significant photoinactivation at that concentration (Figure 2.S4B).

In order to probe whether DIMPy-BODIPY mediates aPDI through, in part, a Type II mechanism for photosensitization, the known singlet oxygen quencher sodium azide was employed in cell survival studies [13]. When the aPDI assay was repeated for A. baumannii in the presence of 0.2–20 mM NaN₃, a statistically significant increase in cell survival was observed for DIMPy-BODIPY (Figure 2.S2, Supplementary Data), suggesting that aPDI of A. baumannii (ATCC-19606) is mediated in part by singlet oxygen production. Cell survival was increased at the sodium azide concentration of 0.2 mM, and the greatest increase was noted at 20 mM: specifically, only 3 log units of inactivation were observed in the presence of the singlet oxygen quencher versus 6 log units in its absence. Above 20 mM, control experiments showed the azide inhibited growth of the bacterium (unpublished data), and for this reason higher concentrations of azide were not pursued due to its toxicity as a metabolic inhibitor.

Despite knowing that in situ formed singlet oxygen is the likely microbiocidal reagent, we are unable to determine if strain-specific variances or species-specific differences are responsible for the different aPDI inactivation efficiencies observed for the Gram-negative bacteria investigated in this study. Given that the photoinactivation of the Gram-positive bacteria was fairly consistent, one obvious factor may be different compositions and properties associated with the bacterial outer membrane of the Gram-negative bacteria. This reasoning could explain, for example, why M. smegmatis was more difficult to photoinactivate when compared to A. baumannii or P. aeruginosa given that its cell wall is thicker than in many other bacteria, as well as more hydrophobic and waxy due to it being rich in mycolic acids/mycolates [31]. Different liposaccharide compositions could also result in variations of the number/density of negative charges present in the outer membrane, which in turn would affect cationic photosensitizer binding, whereas differences in the porins may also affect photosensitizer uptake. Finally, variations in the number and efficiency of efflux pumps, particularly important for antibiotic resistance in K. pneumoniae [32], may also be a factor in expelling photosensitizers that may be uptaken by the bacteria.
2.3.3 Antifungal photodynamic inactivation studies

Two opportunistic Candida pathogens, the *C. albicans* strain ATCC-90028 (Figure 2.5A) and the *C. glabrata* strain ATCC-15545 (Figure 2.5B), proved to be similar in their susceptibilities to photoinactivation with DIMPy-BODIPY. Each strain showed detection limit inactivation (5 log units CFU reduction) with 5 μM DIMPy-BODIPY and either 15 or 30 minutes of illumination (*P*<0.001). For studies performed at 1 μM concentration, the *C. albicans* strain exhibited 69% cell inactivation after 15 minutes of illumination (*P*<0.001), and proved highly susceptible upon an increase in illumination time, with a full 5 log units of inactivation to the detection limit with 30 minutes illumination. The *C. glabrata* strain was 95% (*P*<0.001) inactivated after 15 minutes illumination, though it only exhibited a slight increase to 3 log units inactivation (*P*<0.001) at the longer 30 minute illumination time. The *C. albicans* strain (99.5%, ~2 log units, *P*<0.001) was also inactivated more efficiently than the *C. glabrata* one (95%, ~1 log unit, *P*<0.001) at 30 minutes of illumination and 500 nM DIMPy-BODIPY concentration. No inactivation of the two *Candida* strains was observed for either 15 or 30 minutes illumination at a photosensitizer concentration of 100 nM. Overall, these results with DIMPy-BODIPY compared favorably against the commercial photosensitizer methylene blue [for *C. albicans*, no inactivation was observed with 1 μM methylene blue, and ~4.5 log units at 5 μM PS concentration (Figure 2.S5A); for *C. glabrata*, ~3 log units reduction at 5 μM (Figure 2.S6A)], and were comparable to the detection limit inactivation observed for TMPyP at 5 μM using either 15 or 30 minutes of illumination for *C. albicans* (Figure 2.S5B) and *C. glabrata* (Figure 2.S6B).
Figure 2.5. Photodynamic inactivation of (A) *Candida albicans* ATCC-90028, (B) *Candida glabrata* ATCC-15545, and (C) *Cryptococcus neoformans* ATCC-64538 as a function of DIMPy-BODIPY concentration and illumination time. Displayed is the % survival of the dark control (♦) and the light treated samples at 0.1 µM (■), 0.5 µM (▲), 1 µM (●), and 5 µM (X). Illumination conditions were as follows: 400-700 nm, 65±5 mW/cm², and either 15 or 30 min (total fluences of 59 and 118 J/cm², respectively). As the plating technique employed to determine % survival did not allow for detection of survival rates of <0.001%, data points below the detection limit were set to 0.001% survival for graphing purposes. The shaded areas correspond to undetectable cell survival with the assay employed. In the cases where error bars cannot be visualized, the error bars themselves were smaller than the marker employed in the plot.

The yeast pathogen *Cryptococcus neoformans* ATCC-64538, whose species gives rise to an estimated one million cases of meningitis resulting in 625,000 deaths worldwide each year [33], proved to be more susceptible to photoinactivation with DIMPy-BODIPY than either of the *Candida* species (Figure 2.5C). Detection limit inactivation (5 log units CFU reduction) was achieved at a photosensitizer concentration of 1 µM with 15 minutes illumination, and at 500 nM with the longer 30 minutes illumination (P<0.001). Remarkably, even at the lowest concentration examined of 100 nM DIMPy-BODIPY, a noteworthy 99.5% (2+ log units, P<0.001) reduction in CFU was observed for this *C. neoformans* strain for the 30 minutes illumination time. Again, these results for DIMPy-BODIPY compare favorably against the benchmark photosensitizers methylene blue (Figure 2.S7A) and TMPyP (Figure 2.S7B), both of which were able to reach detection limit inactivation, but required a 5-fold higher concentration of 5 µM to do so when compared with DIMPy-BODIPY.
The difference in photoinactivation efficacy between the three different yeast strains may be attributed to differences in their extracellular structures. *C. neoformans* is encapsulated and known to produce melanin, while Candida species do not. The capsule contains highly negative charged polysaccharides found immediately outside the cell wall [34-36], namely glucuronoxylomannan (GXM), galactoxylomannan (GalXM) and mannoprotein, where GXM makes up approximately 90% of the capsule composition and ranges from 1 to 50 µm in thickness [37]. Both the polysaccharide and melanin production result in a strong negative charge on the cell surface. The high photodynamic inactivation efficacy of the cationic DIMPy-BODIPY towards *C. neoformans* may be explained by a stronger electrostatic interaction between the cell surface and the photosensitizer than with the Candida species.

2.4 Experimental Section

**Materials:** Buffer salts and methylene blue were purchased from Fisher Scientific, Nutrient Broth #234000 was obtained from BD Difco, LB broth Miller from EMD Chemicals, tetramethylpyridyl porphyrin tetratosylate from Frontier Scientific, and Tryptic Soy Broth from Teknova. Polylysine solution was purchased from Sigma Aldrich. Unless otherwise specified, all other chemicals were obtained from commercial sources in the highest purity available. Ultrapure water used for all media and buffers was provided by an Easypure II system (Barnstead). UV-visible absorption measurements were performed on a Varian Cary 50 Bio instrument or a Genesys 10 UV scanning spectrophotometer from Thermo Electron Corp for single wavelength measurements. The photosensitizer 2,6-diiodo-1,3,5,7-tetramethyl-8-(N-methyl-4-pyridyl)-4,4’-difluoroboradiazaindacene [DIMPy-BODIPY; log P = -1.96; \( \lambda_{\text{max}} \) (H\(_2\)O) = 509 nm (75.9 mM\(^{-1}\)cm\(^{-1}\)] was synthesized per published protocol [21,25,38].

**Cell culture:** All bacteria were grown in 5 mL cultures incubated at 37 °C on an orbital shaker under the following growth conditions: *Acinetobacter baumannii* (ATCC-19606) was grown in Miller-LB media without antibiotics; the multi-drug resistant strain of *Acinetobacter baumannii* (ATCC-1605) was grown in Miller-LB media with 5 µg/mL tetracycline;
methicillin-susceptible *Staphylococcus aureus* 2913 was grown in tryptic soy broth media without antibiotics; methicillin-resistant *Staphylococcus aureus* (ATCC-44) was grown in tryptic soy broth media with 5 µg/mL tetracycline; *Pseudomonas aeruginosa* (ATCC-97) was grown in BD Difco Nutrient Broth #234000 with 5 µg/mL tetracycline; *Mycobacterium smegmatis* mc²155 was grown in BD Difco 7H9 media with ADS and 100 µg/mL cycloheximide; *Klebsiella pneumoniae* (ATCC-2146) was grown in BD Difco Nutrient Broth #234000 with 100 µg/mL ampicillin. The vancomycin-resistant strain of *Enterococcus faecium* (ATCC-2320) was grown in BD Difco Bacto Brain Heart Infusion 237500 with 100 µg/mL ampicillin. Each bacterium was grown to a concentration of 1–4 × 10⁸ CFU/mL (determined spectrophotometrically from growth curves using a Genesys 10 UV scanning spectrophotometer) prior to being pelleted by centrifugation (10 min, ~3700 g). Once pelleted, the supernatant was decanted and the cells were resuspended in 5 mL of PBS (170 mM NaCl, 3.4 mM KCl, 10.0 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.2) containing 0.05% Tween-80.

*Candida albicans* (ATCC-90028) and *Candida glabrata* (ATCC-15545) were grown aerobically overnight in yeast extract-peptone-dextrose (YPD) broth at 37 °C. *Cryptococcus neoformans* (ATCC-64538) was grown aerobically in Sabouraud dextrose broth at 30 °C for 48 hours. Cells were harvested by centrifugation (10 min, ~3700 g) and washed twice with PBS. The cells were resuspended in PBS and diluted to ~10⁷ CFU mL⁻¹ (determined spectrophotometrically).

**Viral Propagation:** Vesicular stomatitis virus (VSV) NJ strain was propagated on Vero cells and titered by plaque assay on Vero cells. Dengue 1 virus was propagated on C6/36 mosquito cells and titered on Vero cells by immunofocus assay. Human adenovirus-5 (HAd-5) was propagated on the human lung carcinoma cell line A549 and titered on the same cells.

**Photodynamic Inactivation Assay:** All photosensitization experiments were performed using a non-coherent light source, PDT light model LC122 (LumaCare, USA), and the fluence rate was measured with an Orion power meter (Orphir Optronics Ltd, Israel). All experiments were conducted in triplicate at a minimum, and statistical significance was
assessed via a two-tailed, unpaired Student’s t-test. Sterile stock solutions of DIMPy-BODIPY were prepared in filter sterilized ultrapure water.

**Bacteria and yeast:** 5 mL cultures were incubated with DIMPy-BODIPY (0.01 – 2.5 μM final concentration as indicated in Figures 2.3 and 2.4 for bacteria, and 0.01 – 5 μM final concentration for yeast) on an orbital shaker in the dark for 5 (bacteria) or 15 (yeast) minutes. After incubation, three 1 mL aliquots of the cell suspension were transferred to a sterile 24-well plate (BD Falcon, flat bottom) and illuminated with visible light (400 – 700 nm) with a fluence rate of 65±5 mW/cm² for a variable period of time (5-60 min) while magnetically stirred. The remaining aliquots of cell culture were kept in the absence of light as the dark control. Studies were repeated in the absence of the photosensitizer as a no compound light control. After illumination, each well was 1:10 serially diluted five times. 10 μL from the undiluted well and from each dilution, as well as from the dark control, were plated and incubated in the dark at 37 °C. Each bacterium was grown on gridded six column square agar plates made with their respective growth media without antibiotics, with the exception of *M. smegmatis*, which was plated on BD Difco 7H10-ADS containing 100 μg/mL cycloheximide. The survival rate was determined from the ratio of CFU/mL of the illuminated solution versus that of the dark control. The minimum detection limit was 100 CFU/mL (based on 10 μL plated from the 1 mL undiluted well). Variations in the concentration of the starter culture (1–4 × 10⁸ CFU/mL for bacteria, and 1–4 × 10⁷ CFU/mL for yeast) resulted in a variation of the detection limit spanning the region of 0.001–0.0001% survival for bacteria, and 0.01-0.001% for yeast, respectively. Samples with PS present but kept in the dark (dark control) and illuminated samples without PS (light control) served as controls.

**Vesicular stomatitis virus:** 10⁷ plaque forming units (PFU) of VSV were incubated with DIMPy-BODIPY (0.01 – 1 μM final concentration) for 5 minutes in the dark prior to 30 min under visible light illumination (400 – 700 nm; 65±5 mW/cm²) in a total volume of 100 mL of MEM supplemented with 10 mM HEPES, 1% FBS and antibiotics. Control experiments were similarly performed in the dark. Aliquots of virus samples were subsequently titered on Vero cells, and the virus concentration was determined by plaque assay (detection limit of 40
PFU/mL). Specifically, samples were set up in biological duplicates, viruses were titered by serial 10-fold dilution on Vero cells in 24-well plates at 37 °C. Plaques were detected by crystal violet staining 48 h after infection. Where virus was detectable, the plaques at dilutions where wells contained between 10-20 plaques were counted for titer determination. At concentrations of DIMPy-BODIPY where the virus sample was largely inactivated it was necessary to use wells containing fewer plaques. The limit of detection was 40 PFU/ml.

Dengue-1 virus: \(6.5 \times 10^5\) plaque forming units (PFU) of the virus were incubated with DIMPy-BODIPY (0.25 – 1 μM final concentration) for 5 minutes in the dark prior to 30 min under visible light illumination (400 – 700 nm; 65±5 mW/cm²) in a total volume of 100 mL of MEM supplemented with 10 mM HEPES, 1% FBS and antibiotics. Control experiments were similarly performed in the dark. Samples set up in biological triplicate were used. Virus was titered by serial 10-fold dilution on A549 cells in 24-well plates at 37 °C for 72 h. Immunofoci were detected with an antibody to the E1A protein and a secondary anti-mouse antibody conjugated to horseradish peroxidase. Where virus was detectable, immunofoci at dilutions where wells contained between 10-20 foci were counted for titer determination. At concentrations of DIMPy-BODIPY where the virus sample was largely inactivated it was necessary to use wells containing fewer plaques. The detection limit was 66 FFU/mL.

Human adenovirus-5: \(6.5 \times 10^5\) plaque forming units (PFU) of the virus were incubated with DIMPy-BODIPY (0.25 – 1 μM final concentration) for 5 minutes in the dark prior to 30 min under visible light illumination (400 – 700 nm; 65±5 mW/cm²) in a total volume of 100 mL of MEM supplemented with 10 mM HEPES, 1% FBS and antibiotics. Control experiments were similarly performed in the dark. Samples set up in biological triplicate were used. Virus was titered by serial 10-fold dilution on A549 cells in 24-well plates at 37 °C for 72 h. Plaques were detected by crystal violet staining 48 h after infection. Where virus was detectable, the plaques at dilutions where wells contained between 10-20 plaques were counted for titer determination. At concentrations of DIMPy-BODIPY where the virus sample was largely inactivated it was necessary to use wells containing fewer plaques. The detection limit was 40 PFU/mL.
2.5 Conclusions

We have now further investigated the ability of BODIPY-based photosensitizers to photoinactivate bacteria, fungi, and viruses using visible light (400-700 nm). Although we cannot rule out strain-specific results given the limited numbers of pathogens explored herein, the results obtained demonstrate that DIMPy-BODIPY is able to mediate the photodynamic inactivation of clinically-relevant microbes, including Gram-positive, Gram-negative, and drug-resistant bacteria, as well as pathogenic yeast and model viruses, at nanomolar concentrations and short illumination times. With minor exceptions, DIMPy-BODIPY performed more efficiently over the entire range of microbes studied when compared to the benchmark standards of methylene blue or TMPyP, thus highlighting the utility of the BODIPY-class of photosensitizers for a broad spectrum of potential aPDI application, particularly when one considers the possible synthetic modifications to this modular scaffold. In addition to their potential for in vivo antimicrobial photodynamic therapy, BODIPY-based chromophores may represent an additional class of photosensitizers for incorporation in photomicrobiocidal materials for the elimination of pathogens from surfaces prior to their transmission to hosts [39], and future studies are planned to demonstrate the potential of this new class of photosensitizer for application as a more broadly applicable anti-infective agent.

2.6 Acknowledgments

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2.7. References


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2.8 Supporting Information

Photobleaching/Illumination Time Dependence Study

The time-dependence of illumination on the photobleaching of DIMPy-BODIPY as determined by the resultant loss in aPDT efficacy was undertaken using drug-susceptible *A. baumannii*. The photosensitizer concentration was held constant at 100 nM while the illumination time was varied between 5 and 60 minutes. As seen in Figure 2.S1, an illumination period of 5 minutes resulted in no statistically significant inactivation. When the illumination time was extended to 15 minutes, ~60 % of the bacteria were inactivated, and when extended to 30 minutes greater than 90 % were inactivated. Extending the illumination time to 45 or 60 minutes however, showed no statistically significant inactivation beyond that of the 30 minute illumination.
Figure 2.S1. DIMPy-BODIPY-mediated photodynamic inactivation of *A. baumannii* as a function of the illumination time. The studies were performed in the presence of 100 nM of the photosensitizer. Displayed is the % survival of the dark control (♦) and the light treated samples (■). Illumination conditions were as follows: 5-60 min, 400-700 nm, 65±5 mW/cm² (total fluences of 20-236 J/cm²). As the plating technique employed to determine % survival did not allow for detection of survival rates of <0.0001%, data points below the detection limit were set to 0.0001% survival for graphing purposes. The shaded areas correspond to undetectable cell survival with the assay employed. In the cases where error bars cannot be visualized, the error bars themselves were smaller than the marker employed in the plot.
Figure 2.S2. DIMPy-BODIPY-mediated photodynamic inactivation of *A. baumannii* as a function of the concentration of the singlet oxygen quencher sodium azide (0.001 – 20 mM). The studies were performed at 0 nM (as dark control, ▲), 250 nM (●), and 500 nM (■) concentrations of the photosensitizer. Illumination conditions were as follows: 30 min, 400-700 nm, 65±5 mW/cm² (total fluence of 118 J/cm²). As the plating technique employed to determine % survival did not allow for detection of survival rates of <0.0001%, data points below the detection limit were set to 0.0001% survival for graphing purposes. The shaded areas correspond to undetectable cell survival with the assay employed. In the cases where error bars cannot be visualized, the error bars themselves were smaller than the marker employed in the plot.
Table 2.S1. Cell or virus survival under illumination-only conditions (light control).

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>Survival (% ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>85 ± 21</td>
</tr>
<tr>
<td>MRSA</td>
<td>93 ± 12</td>
</tr>
<tr>
<td>VRE</td>
<td>106 ± 24</td>
</tr>
<tr>
<td><em>Acinetobacter baumannii</em></td>
<td>123 ± 18</td>
</tr>
<tr>
<td>MDRAB</td>
<td>76 ± 28</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>89 ± 9</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>79 ± 8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Virus</th>
<th>Survival (% ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dengue</td>
<td>66 ± 17</td>
</tr>
<tr>
<td>HAd-1</td>
<td>107 ± 23</td>
</tr>
<tr>
<td>VSV</td>
<td>103 ± 22</td>
</tr>
</tbody>
</table>
Figure 2.S3. Photodynamic inactivation of bacteria as a function of TMPyP concentration. (A) Gram positive species. Displayed is the % survival of the dark control (●) and the light treated samples for methicillin-susceptible *S. aureus* (MSSA) ATCC-2913 (■), methicillin-resistant *S. aureus* (MRSA) ATCC-44 (▲), and the vancomycin-resistant *Enterococcus faecium* (VRE) ATCC-2320 strain (x). (B) Gram negative species. Displayed is the % survival of the dark control (●) and the light treated samples for *A. baumannii* ATCC-19606 (■), multidrug-resistant *A. baumannii* (MDRAB) ATCC-1605 (▲), *P. aeruginosa* ATCC-97 (x), and *K. pneumoniae* ATCC-2146 (●). For all bacteria, the illumination conditions were as follows: 30 min, 400-700 nm, 65±5 mW/cm² (total fluence of 118 J/cm²). As the plating technique employed to determine % survival did not allow for detection of survival rates of <0.0001%, data points below the detection limit were set to 0.0001% survival for graphing purposes. The shaded areas correspond to undetectable cell survival with the assay employed. In the cases where error bars cannot be visualized, the error bars themselves were smaller than the marker employed in the plot.
Figure 2.S4. Photodynamic inactivation of bacteria as a function of methylene blue concentration. (A) Gram positive species. Displayed is the % survival of the dark control (♦) and the light treated samples for methicillin-susceptible *S. aureus* (MSSA) ATCC-2913 (■), methicillin-resistant *S. aureus* (MRSA) ATCC-44 (▲), and the vancomycin-resistant *Enterococcus faecium* (VRE) ATCC-2320 strain (x). (B) Gram negative species. Displayed is the % survival of the dark control (♦) and the light treated samples for *A. baumannii* ATCC-19606 (■), multidrug-resistant *A. baumannii* (MDRAB) ATCC-1605 (▲), *P. aeruginosa* ATCC-97 (x), and *K. pneumoniae* ATCC-2146 (○). For all bacteria, the illumination conditions were as follows: 30 min, 400-700 nm, 65±5 mW/cm² (total fluence of 118 J/cm²). As the plating technique employed to determine % survival did not allow for detection of survival rates of <0.0001%, data points below the detection limit were set to 0.0001% survival for graphing purposes. The shaded areas correspond to undetectable cell survival with the assay employed. In the cases where error bars cannot be visualized, the error bars themselves were smaller than the marker employed in the plot.
Figure 2.S5. Photodynamic inactivation of *Candida albicans* ATCC-90028 as a function of photosensitizer concentration and illumination time for A) methylene blue and B) TMPyP. Displayed is the % survival of the dark control (♦) and the light treated samples at 0.1 μM (■), 1 μM (▲), and 5 μM (●). Illumination conditions were as follows: 400-700 nm, 65±5 mW/cm², and either 15 or 30 min (total fluences of 59 and 118 J/cm², respectively). As the plating technique employed to determine % survival did not allow for detection of survival rates of <0.001%, data points below the detection limit were set to 0.001% survival for graphing purposes. The shaded areas correspond to undetectable cell survival with the assay employed. In the cases where error bars cannot be visualized, the error bars themselves were smaller than the marker employed in the plot.
Figure 2.6. Photodynamic inactivation of *Candida glabrata* ATCC-15545 as a function of photosensitizer concentration and illumination time for A) methylene blue and B) TMPyP. Displayed is the % survival of the dark control (♦) and the light treated samples at 0.1 μM (■), 1 μM (▲), and 5 μM (●). Illumination conditions were as follows: 400-700 nm, 65±5 mW/cm², and either 15 or 30 min (total fluences of 59 and 118 J/cm², respectively). As the plating technique employed to determine % survival did not allow for detection of survival rates of <0.001%, data points below the detection limit were set to 0.001% survival for graphing purposes. The shaded areas correspond to undetectable cell survival with the assay employed. In the cases where error bars cannot be visualized, the error bars themselves were smaller than the marker employed in the plot.
Figure 2.S7. Photodynamic inactivation of *Cryptococcus neoformans* ATCC-64538 as a function of photosensitizer concentration and illumination time for A) methylene blue and B) TMPyP. Displayed is the % survival of the dark control (♦) and the light treated samples at 0.1 μM (■), 1 μM (▲), and 5 μM (●). Illumination conditions were as follows: 400-700 nm, 65±5 mW/cm², and either 15 or 30 min (total fluences of 59 and 118 J/cm², respectively). As the plating technique employed to determine % survival did not allow for detection of survival rates of <0.001%, data points below the detection limit were set to 0.001% survival for graphing purposes. The shaded areas correspond to undetectable cell survival with the assay employed. In the cases where error bars cannot be visualized, the error bars themselves were smaller than the marker employed in the plot.
Chapter 3
Porphyrin-Cellulose Nanocrystals: A Photobactericidal Material that Exhibits Broad Spectrum Antimicrobial Activity

Bradley L. Carpenter,¹ Elke Feese,¹ Hasan Sadeghifar,² Dimitris S. Argyropoulos,¹²³ and Reza A. Ghiladi *¹

¹ Department of Chemistry, North Carolina State University, Raleigh, North Carolina, 27695-8204
² Department of Forest Biomaterials, North Carolina State University, Raleigh, North Carolina, 27695-8005
³ Department of Chemistry, University of Helsinki, Helsinki Finland


Bradley L. Carpenter contributed the antimicrobial studies and characterization as well as porphyrin synthesis, and cellulose nanocrystal preparations. Elke Feese assisted in porphyrin synthesis. Hasan Sadeghifar contributed the cellulose nanocrystal preparations.
3.1 Abstract

Towards our overall objectives of developing potent antimicrobial materials to combat the escalating threat to human health posed by the transmission of pathogenic bacteria that adhere to surfaces, we have investigated the photobactericidal activity of cellulose nanocrystals that have been modified with a porphyrin-derived photosensitizer. The ability of the resulting porphyrin-cellulose nanocrystals, CNC-Por (1), to mediate bacterial photodynamic inactivation was investigated as a function of bacterial strain, incubation time, and illumination time. Despite forming an insoluble suspension, CNC-Por (1) showed excellent efficacy toward the photodynamic inactivation of Acinetobacter baumannii, multidrug-resistant Acinetobacter baumannii (MDRAB), and methicillin-resistant Staphylococcus aureus (MRSA), with the best results achieving 5-6 log units reduction in colony forming units (CFUs). The porphyrin-cellulose nanocrystals were also capable of mediating the inactivation of Pseudomonas aeruginosa, although at reduced activity (2-3 log units reduction in CFUs). Imaging of CNC-Por (1) using confocal laser scanning microscopy after incubation with A. baumannii suggested a lack of internalization of the photosensitizer. Further research into alternative materials such as CNC-Por (1) may lead to their application in hospitals and healthcare-related industries wherein novel materials with the capability of reducing the rates of transmission of a wide range of bacteria, particularly antibiotic resistant strains, are desired.
3.2 Introduction

The increasing prevalence of antibiotic-resistant bacteria has led to a rapidly emerging need to develop new strategies to mitigate nosocomial infections which are mediated by the survival of these pathogenic bacteria on surfaces, ultimately leading to a rise in incidence rates of infectious diseases. Such hospital acquired infections (HAIs) now infect 1 out of every 20 hospitalized patients, and as the cause of approximately 100,000 deaths annually in the United States alone, represent the sixth leading cause of mortality in the US and an increasing economic burden on an already strained healthcare system \(^1\). The pathogens that give rise to HAIs have are capable of surviving for prolonged periods in hospital environments (linens, drapes, bed rails) and on the hands of healthcare workers \(^2\). Depending on the strain, *Acinetobacter baumannii*, *Mycobacterium tuberculosis*, and *Staphylococcus aureus* all have been shown to remain viable on dry, abiotic surfaces for upwards of 4-7 months \(^3\). Surfaces that are not effectively disinfected can therefore lead to the proliferation of pathogens and their subsequent transmission to new hosts, thereby necessitating research into the next generation of materials that possess antimicrobial properties.

Antimicrobial photodynamic therapy (aPDT) \(^4\), also referred to as photodynamic antimicrobial chemotherapy (PACT) \(^5\), has emerged as an alternative strategy for the treatment of microbial infections. aPDT employs a light-activated photosensitizer that generates bactericidal species (i.e., radicals or singlet oxygen, \(^1\)O\(_2\)) upon illumination with visible or near infrared light. Singlet oxygen possesses a number of unique characteristics that make it amenable for antimicrobial applications. These include damaging reactivity with most biomolecules \(^6\), a short lifetime of \(\sim 10^{-6}\) s in aqueous environments \(^7\), and the formation of a harmless byproduct when left unreacted (ground state molecular oxygen). Additionally, development of bacterial resistance against aPDT is believed to be unlikely due to the unspecific damage caused by singlet oxygen \(^4b\), making it a particularly attractive for treatment for bacterial infections. While significant efforts have focused on the application of aPDT for the treatment of bacterial infections *in vivo*, a number of more recent studies have demonstrated that photobactericidal materials may have widespread applicability for the elimination of
bacteria prior to infection. Specifically, it has been shown that photosensitizers are capable of mediating the photodynamic inactivation (PDI) of bacteria when incorporated, either through covalent or non-covalent conjugation, into a number of different materials (films, membranes, polymers, nanoparticles, and fabrics). Singlet oxygen is able to diffuse over distances as long as 100-200 nm in water and upwards of 1 mm in air, allowing the potential for the effective inactivation of bacteria on both wet and dry surfaces when the photosensitizer is within sufficient proximity to the pathogen. Photobactericidal surfaces generated from the covalent attachment of photosensitizers to a solid support may possess a number of advantages over other antimicrobial materials that incorporate compounds derived from biomolecules (antimicrobial peptides, natural products), cationic species (quaternary ammonium, pyridinium, or phosphonium salts), organic compounds (conventional antibiotics, phenolics, N-halamines, biguanides), or heavy metals (copper, silver, zinc). These advantages include employing an environmentally benign biocidal agent in singlet oxygen, permanent attachment of the photosensitizer through covalent conjugation, and the ability of the photosensitizer to function in the absence of direct contact with the bacterium. Additionally, as singlet oxygen causes non-specific damage, photodynamic materials may also possess antiviral, antifungal, and antiparasitic properties.

As a potential solid support for photobactericidal materials, cellulose possesses a number of chemical and physical properties that have been well characterized. It is the most naturally abundant biomaterial on the planet, and represents a structural motif upon which the bulk material of our plant systems and a number of derivative biopolymers (e.g., chitin) are based. Cellulose is comprised of very regular crystalline units or nanodomains that, upon acid hydrolysis, yield highly crystalline rod-like hydrophilic particles at the nanoscale level (100-400 nm in length) that possess high mechanical strength and enhanced properties when compared to other polymers. For example, these cellulose nanocrystals (CNC) possess a high melting temperature that may positively affect the thermal transition properties of any covalently attached functional groups on them, a very attractive proposition in designing high melt composites, thermal extrusions, and temperature-resistant materials. While cellulose
nanocrystals have been explored mainly for their contributions to macroscopic properties of bulk materials, they also possess a number of unique molecular characteristics of significance that allow them to act as scaffolds for nanomaterials. These include: i) taking the form of rigid molecular rods of well-defined dimensions; ii) enantiopurity with embedded polymeric directionality; iii) an etched molecular pattern on their surfaces composed of primary hydroxyl groups at the C6 position which are amenable to chemical modification. Thus, cellulose is a readily abundant, renewable material that, when combined with these attractive chemical features in one system, provides a unique paradigm for chemical transformations and functionality in the design of novel photobactericidal materials.

Previously, we described the synthesis and characterization of CNC-Por (I), a photobactericidal material formed from the covalent attachment of an alkyne-containing porphyrin photosensitizer to the surface of azide-modified cellulose nanocrystals using the Cu(I)-catalyzed Huisgen-Meldal-Sharpless 1,3-dipolar cycloaddition reaction (Figure 3.1). While the focus of that report was primarily on the physico-chemical properties of CNC-Por (I), we also demonstrated that this material was able to mediate the photodynamic inactivation of both methicillin-susceptible S. aureus (MSSA) and M. smegmatis (as a surrogate for M. tuberculosis) with high efficiency, yet exhibited relatively low bactericidal activity against E. coli. Given the limited scope of the previous antimicrobial study in which only three strains of drug-susceptible bacteria were explored, and in order to gain insight into the mechanism of porphyrin-cellulose nanocrystals as photosensitizers in the photoinactivation of bacteria, the focus of the present report is to further explore the potential of CNC-Por (I) as a photobactericidal material. In addition to supplemental characterization of I by UV-visible spectroscopy, the antimicrobial properties of this material were investigated against Acinetobacter baumannii, multiple-drug resistant Acinetobacter baumannii (MDRAB), methicillin-resistant Staphylococcus aureus (MRSA), and Pseudomonas aeruginosa. As will be demonstrated, CNC-Por (I) exhibited excellent antimicrobial activity for all strains examined with the exception of P. aeruginosa, which was only moderately inactivated under the conditions employed. When combined with the results of the previous investigation, the
current study strongly suggests that CNC-Por (1) is able to mediate the inactivation of bacteria across order, class, and phylum, highlighting the potential application of this photobactericidal material in eradicating microbes from contaminated surfaces given its apparent broad spectrum antimicrobial activity. Additionally, we imaged *A. baumannii* using confocal fluorescence microscopy in the presence of both CNC-Por (1) and the solution-based photosensitizer Zn-EpPor (3), the results of which will be discussed in the context of photosensitizer internalization and the mode of action of CNC-Por (1) in mediating antimicrobial PDI.

**Figure 3.1.** Synthesis of cellulose nanocrystal-porphyrin conjugate CNC-Por (1).

### 3.3 Materials and Methods

**Materials:** Buffer salts were purchased from Fisher Scientific, Nutrient Broth #234000 was obtained from BD Difco, LB broth Miller from EMD Chemicals, and Tryptic Soy Broth from Teknova. Polylysine solution was purchased from Sigma Aldrich. Unless otherwise specified, all other chemicals were obtained from commercial sources in the highest purity available. Ultrapure water used for all media and buffers was provided by an EasyPure II system (Barnstead). UV-visible absorption measurements were performed on a Varian Cary 50 Bio instrument or a Genesys 10 UV scanning spectrophotometer from Thermo Electron Corp for
single wavelength measurements. High precision no. 1.5 coverslips were purchased from Bioscience Tools. All procedures were carried out under commonly practiced sterile techniques. ICP-mass spectrometry was provided by the Environmental and Agricultural Testing Service (EATS) Facility (NC State University).

**Preparation of CNC-Por (1):** Porphyrin-cellulose nanocrystals, CNC-Por (1), and its benzoylated derivative, 1-Bz, were prepared as previously described. ICP-mass spectrometry was used to determine the concentration of Zn ions in samples of CNC-Por (1), which is equivalent to the porphyrin loading if complete metallation of the porphyrin is assumed. 10 mg of lyophilized CNC-Por (1) was digested in 10 mL of HCl, followed by heating to 60 °C for ~2 hours. Once digested, it was assumed that the porphyrin was fully demetallated, allowing for the Zn concentration to be determined using ICP mass spectrometry. The porphyrin loading was found to be 0.062 μmoles porphyrin/mg CNC-Por (1). Using this empirically determined value, a 1 mM stock solution was prepared using filter sterilized ultrapure water, and was stored in the dark at 4 °C for subsequent use in the PDI assays.

**UV-Visible Spectroscopic Characterization of CNC-Por (1):** UV-visible absorption spectra were collected at room temperature with a Cary 50 UV-visible spectrophotometer using quartz microcuvettes (1 cm pathlength). A stock solution of 1-Bz and one of the precursor porphyrin Zn-EpPor (3) were prepared in DMSO. After dilution with water to a final solution composition of 30:1 H₂O:DMSO, the UV-visible spectra were recorded. The known molar absorption coefficient of compound Por (195 mM⁻¹cm⁻¹) was used to normalize the spectra.

**Bacterial Cell Culture:** The bacterial strains *Acinetobacter baumannii* (ATCC #19606), multi-drug resistant *Acinetobacter baumannii* (MDRAB; ATCC #BAA-1605), and methicillin-resistant *Staphylococcus aureus* (MRSA; ATCC #BAA-44) were kindly donated by Prof. Christian Melander (North Carolina State University). *Pseudomonas aeruginosa* (ATCC #97) was obtained from the American Type Culture Collection (Manassas, VA, USA). Aliquots of each bacterial species were prepared in a 1:1 broth:glycerol ratio and stored at -80 °C. *Acinetobacter baumannii* was grown in Miller LB broth without antibiotics, while the
MDRAB strain was grown in Miller LB broth with 5 µg/mL tetracycline. Methicillin-resistant *S. aureus* was grown in Tryptic-Soy broth with 5 µg/mL tetracycline, and *Pseudomonas aeruginosa* was grown in BD Difco Nutrient Broth (#234000) with 5 µg/mL tetracycline. All bacterial inoculations were incubated on a shaker at 37 °C and 500 rpm. Growth curves for each strain were prepared under these conditions.

**Incubation of Cells with Photosensitizer:** Each 5 mL culture with a concentration of 1-3×10^8 CFU/mL (determined spectrophotometrically from the growth curve) was pelleted by centrifugation (10 min, ~3700 x g) at 4 °C, the supernatant discarded, and the cells were resuspended in a total volume of 5 mL PBS (phosphate buffered saline = 170 mM NaCl, 3.4 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.2 with additional 0.05 % Tween 80). A 1 mL aliquot of the bacterial solution was transferred to a separate culture tube to serve as a compound-free control. An appropriate volume of the CNC-Por (I) photosensitizer stock solution was added to the remaining 4 mL bacterial culture for a final concentration of 20 µM. The two tubes were wrapped in aluminum foil and were incubated for 5, 15, 30 or 60 minutes in the dark at room temperature and agitated by a vortex mixer set to the second lowest speed. Once the incubation was complete, three 1 mL aliquots from the culture tube containing the photosensitizer were placed in separate wells on a 24-well plate. The compound-free control (no compound control) and the remaining 1 mL culture containing CNC-Por (I) (PS treated dark control) were placed in the dark while the plate underwent illumination.

**Cell Illumination Conditions:** All photosensitization experiments were performed using a noncoherent light source, PDT light model LC122 (LumaCare, USA), and the fluence rate was measured with an Orion power meter (Orphir Optronics Ltd., Israel) to measure intensity above and below the plate. 1 mL aliquots of the cell suspension in PBS were added to a sterile 24 well plate (BD Falcon, flat bottom) and illuminated with visible light (400-700 nm) with a fluence rate of 65 mW/cm² for the duration of 15 or 30 minutes (corresponding to fluences of 59 or 118 J/cm²) while magnetically stirred (200 rpm). Tween-80 (0.05%) was added to the buffer to prevent excessive aggregation of the PS during the illumination process. After
illumination, an aliquot was used for viability assays. All cell illumination experiments were conducted in triplicate at a minimum.

**Cell Survival Assays:** Once illumination was complete, 40 µL from each illuminated cell suspension, compound-free dark control, and compound-containing dark control, were 1:10 serially diluted in PBS six times and plated on square plates (LB Broth Miller-Agar plates for both *A. baumannii* and MDRAB, Difco Nutrient Broth-Agar plates for *P. aeruginosa*, and Tryptic Soy Broth-Agar plates for methicillin-resistant *S. aureus*) as described by Jett and coworkers. The plates were incubated at 37 °C in the dark. The survival rate was determined from the ratio of CFU/mL of the illuminated solution and the no compound control. Due to the plating technique employed, a maximum of a 6 log unit change in CFU/mL corresponding to \( \geq 100 \) CFU/mL could be detected for an initial concentration of \( 1 \times 10^8 \) CFU/mL. Survival rates of \(< 0.0001\%\) could not be detected. The percentage of survival for samples for which the corresponding plates did not show any colonies was thus set to the detection limit of 0.0001\%. Samples with photosensitizer present but kept in the dark (dark control) served as a control. Statistical significance was assessed via a two-tailed, unpaired Student’s t-test.

**Confocal Fluorescence Microscopy of CNC-Por (1):** Confocal fluorescence microscopy was performed on a Zeiss 710 LSM (Cellular and Molecular Imaging Facility, NCSU). All experiments were performed with *Acinetobacter baumannii* at concentrations comparable to the cell survival assays. Bacterial samples were prepared with and without photosensitizer, using either the precursor porphyrin Zn-EpPor (3) in solution or CNC-Por (1) as the photosensitizer. All concentrations, incubation times, and illumination times were comparable to those performed during the PDI survival assays. In order to eliminate as many variables as possible, two *A. baumannii* samples were grown simultaneously in culture tubes until reaching 1-3×10^8 CFU/mL. The bacteria were pelleted via centrifugation and resuspended in PBS buffer. CNC-Por (1) was then added to one culture tube, while Zn-EpPor (3) was added to the other at equal concentration. The tube with CNC-Por (1) as the photosensitizer was placed on a vortexer in the dark for 30 minutes, while the tube containing the Por photosensitizer was
placed on an orbital shaker (400 rpm) at 37 °C also in the dark. A 1 mL aliquot of the PS-incubated cells was transferred to a well on a 24-well plate and illuminated for 30 minutes, after which a sample from each well was then prepared per standard microscopy protocols and imaged. Excitation was performed using an Argon laser at 458 nm at a quarter of its maximal intensity, and the intrinsic porphyrin fluorescence was observed. All images were obtained under identical conditions (pinhole, gain, filters) for comparison purposes.

3.4 Results

Preparation and UV-visible spectroscopic characterization of CNC-Por (1)

CNC-Por (1) and its benzoylated derivative, 1-Bz, were prepared as previously described (Figure 3.1). Briefly, cellulose nanocrystals (CNC, 2) were obtained from the acid hydrolysis of cotton fibers and were subsequently treated with tosyl chloride followed by sodium azide to modify the primary hydroxyl groups for installment of surface-azide units. Covalent attachment of the alkyne-containing porphyrin Zn-EpPor (3) to the azide-modified cellulose nanocrystals was performed using the Cu(I)-catalyzed Huisgen-Medal-Sharpless 1,3-dipolar cycloaddition reaction. Purification of the target compound was performed as published, resulting in the formation of CNC-Por (1) as an insoluble, green crystalline material.

Treatment of 1 with benzoyl chloride in the ionic liquid 1-allyl-3-methylimidazolium chloride resulted in the benzoylated derivative of CNC-Por, 1-Bz, thus rendering the normally insoluble cellulose material soluble in organic and aqueous solvents for subsequent characterization by UV-visible spectroscopy (Figure 3.2). The electronic absorption spectrum of 1-Bz in H$_2$O:DMSO (30:1) exhibits spectral features [UV-visible: 442 (Soret), 567, 612 nm] that are typical for a Zn-metallated porphyrin of the tris-(4-methylpyridin-4-ium-1-yl) scaffold such as that employed in 1. When compared to the spectrum of Zn-EpPor (3) [UV-visible: 436 (Soret), 564, 608 nm] obtained under identical conditions, 1-Bz exhibits bathochromic shifts that are likely attributable to the differences in the local environment (e.g., polarity and solvation) of the porphyrin due to the presence of the covalently appended cellulose. Notably,
the free-base (unmetallated) precursor, EpPor, exhibits spectral features [UV-visible: 426 (Soret), 521, 557, 591, 648 nm] that are distinct from 1-Bz and its Zn-metallated analog under similar conditions. As 1-Bz does not exhibit a hypsochromic shift of its Soret band that is indicative of the free-base porphyrin, the UV-visible spectroscopic data strongly support a fully metallated porphyrin (i.e., 1:1 Zn:porphyrin stoichiometry) in CNC-Por (1).

**Figure 3.2.** Electronic absorption spectra of benzoylated CNC-Por (1-Bz) (red) and the water-soluble precursor porphyrin Zn-EpPor (3) (black) in 30:1 H2O:DMSO solution.

**Photodynamic inactivation studies with CNC-Por (1)**

The photobacterial activity of CNC-Por (1) was investigated using *A. baumannii*, multidrug resistant *A. baumannii* (MDRAB), methicillin-resistant *S. aureus*, and *P. aeruginosa*. For the photodynamic inactivation studies, the bacteria were grown to a concentration of \( \sim 10^8 \) colony forming units per mL (CFU/mL), resuspended in the illumination buffer (0.05% Tween 80 in PBS), incubated with 20 \( \mu \)M (based on porphyrin loading, see below) of CNC-Por (1) in the dark for the corresponding incubation time, and illuminated with white light (400-700 nm) with a fluence rate of 65 mW/cm\(^2\) for 15 or 30 minutes (corresponding to fluences of 59 or 118 J/cm\(^2\), respectively). As supported by the UV-visible characterization of the porphyrin-
cellulose nanocrystals (*vide supra*), a 1:1 zinc-to-porphyrin stoichiometry was assumed which enabled the porphyrin loading of the stock solution of 1 to be determined by a direct measurement of zinc ion concentration using ICP-mass spectrometry. Thus, the 20 μM concentration of CNC-Por (1) employed refers to the concentration of the porphyrin on 1 present in the suspension. Control experiments included survival assays of the bacteria in PBS buffered solution kept in the dark in the absence (no compound dark control) and presence (PS treated dark control) of 1. The percentage of cell survival was calculated as the ratio of the colony count from PS-treated illuminated cultures and the no compound dark control, the latter being set to 100% survival in Figures 3.3-3.5.

*Acinetobacter baumannii* and MDRAB. The results for the photodynamic inactivation of *A. baumannii* and MDRAB are shown in Figures 3.3A and 3.3B, respectively. For the PS treated dark control, *A. baumannii* exhibited upwards of 0.4 log units reduction in CFUs (*P* = 0.002), indicative of a minor amount of dark toxicity of 1 against this bacterium (Figure 3.3A). Upon illumination for 15 minutes, however, a more significant and clear loss in viable cells for *A. baumannii* was noted. Although some variation in the data was observed, overall a 4-5 log units reduction in CFUs was achieved (*P* < 0.0001), with the results appearing to be independent of the dark incubation time (5-60 min). With the exception of the 15 minute dark incubation time, increasing the illumination period to 30 minutes resulted in an improvement in the inactivation of *A. baumannii* to 4.5-5.5 log units reduction in viable cells. Comparison of the 30 minute illumination data again revealed no statistically significant dependence of survival as a function of dark incubation time.

MDRAB exhibited dark toxicity (upwards of ~80%) that was more apparent at incubation times greater than 15 minutes (Figure 3.3B). As was observed for *A. baumannii*, illumination of the MDRAB culture for 15 minutes resulted in 4 log units reduction in CFUs (*P* < 0.0001) for the 5 minute dark incubation, and greater than 5 log units reduction in viable cells when the dark incubation time was further lengthened (15-60 min). A statistically significant increase in inactivation by ~1 log unit more was observed when the illumination time was increased to 30 minutes. With the exception of the results for the 5 minute dark
incubation data, which were virtually identical between the two bacteria, MDRAB was slightly more susceptible to photodynamic inactivation by CNC-Por (1) when compared to A. baumannii.
Figure 3.3. Photodynamic inactivation of A) *A. baumannii* and B) MDRAB using 20 µM CNC-Por (1) as the photosensitizer. The dark incubation time was varied over 5, 15, 30 and 60 min. Displayed is the % survival in PBS buffered bacterial suspensions for the compound-free dark control (■), CNC-Por dark control (■) and light treated samples after illumination times of 15 (□) or 30 (△) minutes (total fluences of 59, and 118 J/cm², respectively, at 65 mW/cm²). As the plating technique employed to determine % survival did not allow for detection of survival rates of <0.0001%, data points below the detection limit are set to 0.0001% survival for graphing purposes. The shaded areas correspond to undetectable cell survival with the assay employed.

Methicillin-resistant *Staphylococcus aureus* (MRSA). The photodynamic inactivation of methicillin-resistant *S. aureus* by CNC-Por (1) was found to be highly effective (Figure 3.4)
under all conditions examined. Regardless of the dark incubation time or the illumination period, no CFUs were detected, and survival rates were therefore below the detection limit of <0.0001%, corresponding to an impressive 6 log units reduction in viable cells ($P < 0.0001$). Dark controls of the PS-treated cultures containing compound 1 showed at most 0.5 log units reduction in CFUs at the longer dark incubation times. *S. aureus*, being a Gram-positive bacterium, lacks an outer cell membrane, and it is likely for this reason that *S. aureus* is so highly susceptible to the cytotoxic species produced during the photodynamic inactivation process.

![Figure 3.4](image.png)

**Figure 3.4.** Photodynamic inactivation of methicillin-resistant *S. aureus* using 20 µM CNC-Por (1) as the photosensitizer. Displayed is the % survival in PBS buffered bacterial suspensions for the compound-free dark control (■), CNC-Por dark control (■) and light treated samples after illumination times of 15 (■) or 30 (■) minutes. Assay conditions were as specified in Figure 3.3.

*Pseudomonas aeruginosa*. In comparison to the other bacterial strains investigated, *P. aeruginosa* appeared to be the least susceptible to photodynamic inactivation by CNC-Por (1) (Figure 3.5). No statistically significant inactivation of *P. aeruginosa* was observed when
incubated for less than 30 minutes. When the incubation was 30 or 60 minutes, a 2.5-3 log units reduction in the cell count was observed for both illumination times ($P < 0.0001$). Thus, the dark incubation time that preceded illumination did appear to play a role in the ability of CNC-Por (1) to mediate the photodynamic inactivation of *P. aeruginosa*, whereas the incubation time had a minimal effect on the ability for 1 to inactivate *A. baumannii*/MDRAB or MRSA. No statistically significant dark toxicity on the survival of *P. aeruginosa* was attributed to CNC-Por (1).

**Figure 3.5.** Photodynamic inactivation of *P. aeruginosa* using 20 µM CNC-Por (1) as the photosensitizer. Displayed is the % survival in PBS buffered bacterial suspensions for the compound-free dark control (■), CNC-Por dark control (■) and light treated samples after illumination times of 15 (■) or 30 (■) minutes. Assay conditions were as specified in Figure 3.3.
Confocal fluorescence microscopy studies of *A. baumannii* with CNC-Por (1)

Confocal fluorescence microscopy was employed to investigate the interaction of CNC-Por (1) with *A. baumannii* (Figure 3.6) prior to and after completion of a typical cell survival assay. Fluorescence was achieved by employing an excitation wavelength of 458 nm that was nearly coincident with the Soret absorbance of CNC-Por (1) (442 nm). Panel A depicts the size distribution of the cellulose-porphyrin nanocrystalline aggregates relative to the bacterium. Although individual cellulose nanocrystals (average length of 100-400 nm \(^{22, 25b}\)) can be observed, the material formed aggregates that were generally much larger (~ 1-8 \(\mu\)m in length).

Notably, as illustrated in Panels B and C, no fluorescence was observed either internalized within *A. baumannii*, or localized to its cell membrane. Although this does not conclusively rule out a photosensitizer internalization mechanism or adherence of the PS to the cell membrane as the mechanism for bacterial PDI, it does suggest that the porphyrin of CNC-Por (1) remains covalently bound to the cellulose throughout the cell illumination assay and does not undergo degradation to form a water-soluble species. By contrast, as shown in Panel D, the water-soluble porphyrin Zn-EpPor (3), when in solution and not bound to the cellulose nanocrystals, led to the fluorescence being localized on *A. baumannii*. Although the resolution of the confocal microscope did not allow for the determination of whether the cationic photosensitizer was internalized by the bacterium, or simply bound through an electrostatic interaction to the negatively charged bacterial cell membrane, the results highlight a stark contrast between how an insoluble PS such as CNC-Por (1) interacts with a bacterium when compared with a more traditional, solution-based photosensitizer.
Figure 3.6. Confocal fluorescence microscopy images of *A. baumannii* acquired post illumination (30 min) in the presence of A-C) CNC-Por (1) and D) the solution-based Por photosensitizer. See text for additional details.

3.5 Discussion

The attractiveness of cellulose as a bioavailable, biocompatible, biodegradable, and renewable scaffold for photobactericidal materials has garnered increasing attention recently, particularly as it is amenable to the covalent attachment of photosensitizers using current bioconjugation methodologies \(^8,^{12,28}\). Our focus has been on utilizing the unique properties and molecular control afforded by cellulose nanocrystals, allowing for well-defined, discrete nanodomains that have been derivatized with a variety of antimicrobial functional groups, and in particular with photosensitizers, thereby allowing for fundamental and systematic investigations of the (photo)bactericidal efficacy of these systems. Based on our earlier work
demonstrating the efficacy of the solution-based cationic photosensitizer TMPyP [meso-tetrakis(1-methyl-4-pyridinyl)porphyrin tetratosylate] in mediating the photodynamic inactivation of *M. smegmatis* \(^{29}\), we chose to covalently attach an alkyne-containing analogue, Zn-EpPor (3) \(^{27}\), to azide-modified cellulose nanocrystals \(^{25b}\) using the Cu(I)-catalyzed Huisgen-Meldal-Sharpless 1,3-dipolar cycloaddition reaction (a “Click” reaction) \(^{30}\), thereby forming the benchmark compound CNC-Por (1) \(^{26}\). Along with characterizing 1 using nuclear magnetic resonance and infrared spectroscopies, as well as thermogravimetric analysis, gel permeation chromatography and other analytical methods, the photobactericidal properties of the porphyrin-cellulose nanocrystals were investigated, but in limited fashion. Our aim here is to provide supporting spectroscopic characterization of CNC-Por (1), and, moreover, to expand the breadth and scope of the antimicrobial study to include additional strains not previously investigated, including antibiotic-resistant ones.

In our previous study of CNC-Por (1) \(^{26}\), the porphyrin loading was determined using atomic absorption spectroscopy, with the critical assumption being that the porphyrin was fully metallated with zinc (i.e., a 1:1 Zn:porphyrin stoichiometry was present). However, if full or even partial demetallation of the porphyrin occurred during the synthesis of CNC-Por (1), this would result in an erroneously low value of porphyrin loading, thereby leading to the use of a higher concentration of 1 in the cell survival assays than desired, and thus an overstatement of the efficacy of the photobactericidal material. Thus, it was imperative to conclusively establish that no detectable demetallation of the porphyrin in CNC-Por (1) occurred during its synthesis and subsequent purification. To that end, CNC-Por (1) was benzoylated for improved solubility \(^{26}\), and its solution UV-visible spectrum was obtained. The electronic absorption spectrum exhibited features that were consistent with a zinc-metallated porphyrin, and, importantly, it was not possible to observe any of the spectroscopic features attributable to a free-base (demetallated) porphyrin (hypsochromic shift of the Soret band and the presence of four Q bands). Given the >10 nm difference in the wavelength maxima of the Soret bands for the metallated and free-base porphyrins \(^{27}\), and given the distinct Q band features for each, we estimate that CNC-Por (1) is minimally 95% metallated.
The extent of antimicrobial activity was examined for CNC-Por (1) as a function of bacterial strain, including *A. baumannii*, MDRAB, methicillin-resistant *S. aureus*, and *P. aeruginosa*. The best results demonstrated a wide range of photobactericidal activity across bacterial order, class and phylum, from a 2.5 log units reduction in viable cells for *P. aeruginosa* (60 min incubation, 30 min illumination, 99.7% cell inactivation, $P < 0.0001$) to a 6 log units reduction for MRSA (all conditions, 99.9999+% cell loss, $P < 0.0001$). As was previously noted for *S. aureus*\(^{26}\), the MRSA strain employed here appeared to be the most susceptible bacterium of those studied for bacterial PDI when mediated by CNC-Por (1). This result is not unexpected, however, as Gram-positive bacteria such as *S. aureus* lack an outer cell membrane, and therefore possess a relatively more permeable cell wall that likely renders the bacterium more susceptible to damage by the cytotoxic species generated from PDI\(^{31}\). In contrast to the excellent results achieved with *S. aureus*, the maximum viable cell loss observed for the Gram-negative *P. aeruginosa*, although still significant at about 99.7%, was ~3.5 log units less, and required the longer incubation periods prior to illumination. One could attribute this observed difference in bacterial inactivation to the presence (Gram-negative) or absence (Gram-positive) of the outer bacterial cell wall. However, the other Gram-negative bacterial strains examined herein, *A. baumannii* and MDRAB, displayed a reduction in viable cells that, although intermediate between *S. aureus* and *P. aeruginosa*, was still nearly to the detection limit (~5-6 log units reduction). Our previous study investigating the inactivation of *E. coli* (Gram-negative) with CNC-Por (1) revealed a ~2 log units reduction in CFUs, whereas *M. smegmatis* (mycobacterium/Gram-positive) was inactivated up to 3.5 log units, and up to 6 log units inactivation was observed for methicillin-sensitive *S. aureus* (MSSA). Thus, while the lack of the outer cell wall likely plays a significant role in the inability of Gram positive bacteria to resist photobactericidal inactivation, other factors that have yet to be determined must also play significant roles in order to explain the variability in inactivation of the Gram negative strains.

The photobactericidal activity of CNC-Por (1) was also examined as a function of light dose and dark incubation time. Not surprisingly, under nearly all conditions examined, the 30
min light dose achieved a greater reduction in viable cells than the 15 min light dose when a statistically significant difference between the two conditions was observed, and is likely attributable to the higher amount of cytotoxic species, in particular singlet oxygen, formed during the increased illumination time. No conclusive cell survival dependence on dark incubation times was observed for A. baumannii or MDRAB. Additionally, due to the complete inactivation of MRSA, no dark incubation time dependence could be observed with the detection limit utilized. Interestingly, however, a dependence on the dark incubation time was observed for P. aeruginosa, as statistically significant inactivation was only observed for the 30 and 60 min incubation times and not for the shorter ones. Previously, we also noted a dark incubation time dependence on the inactivation of M. smegmatis by CNC-Por (1), and at that time it was hypothesized that the action of putative endogenous mycobacterial cellulases may partially degrade CNC-Por (1), leading to the release of smaller, water-soluble porphyrin-polysaccharides that could lead to the observed increase in PDI efficiency at longer dark incubation times. However, to the best of our knowledge, there are no known cellulases of P. aeruginosa. A combination of other factors, such as preferential adherence of one bacterium over another to the porphyrin-cellulose nanocrystals due to the differences in bacterial cell wall morphology, or possibly even the secretion of exopolysaccharide biofilms by specific bacteria, could lead to the observed dark incubation time dependencies. Additional studies are currently underway in our laboratory to elucidate the origin of the dark incubation time dependence on the antimicrobial PDI of P. aeruginosa and M. smegmatis.

In order to gain more insight into the bactericidal mechanism of CNC-Por (1), confocal fluorescence microscopy was employed to image the PS-treated cultures of A. baumannii. Qualitatively, both the bacterium and the porphyrin-cellulose nanocrystals were indistinguishable pre- and post-illumination, suggesting that the morphology of the nanocrystalline material was unchanged despite having undergone a photodynamic process. Importantly, neither pre- nor post-illuminated samples when treated with CNC-Por (1) exhibited evidence of porphyrin fluorescence either internalized within A. baumannii, or localized on the bacterial cell surface. By contrast, when A. baumannii was treated with the
water-soluble Zn-EpPor (3), fluorescence was observed superimposed upon the bacterium, suggesting that the solution-based photosensitizer was either internalized or bound to the cell membrane. Taken together, the results support the hypothesis that direct binding and uptake of the photosensitizer are not necessary requirements for photodynamic inactivation by CNC-Por (1), and, as singlet oxygen has been shown to diffuse over distances as long as 100-200 nm in aqueous solution \(^1\), the production of singlet oxygen or other cytotoxic species in close proximity to the bacteria may be sufficient to result in cell death. By the same token, in light of our previous observation that a 100-500 fold higher porphyrin concentration is needed for CNC-Por (1) to photoinactivate \textit{M. smegmatis} to the same extent when compared to soluble photosensitizers [i.e., Zn-EpPor (3)] \(^{26,29}\), one can regard a diffusion-based process of cytotoxic species from the insoluble photosensitizer as being less effective than a direct interaction (internalization or localization of the PS to the bacterial cell membrane), the latter being what may be needed for effective aPDT at very low photosensitizer concentrations \textit{in vivo}.

3.6 Conclusions

We have now further investigated the ability of CNC-Por (1) to photoinactivate bacteria of different genera using visible light (400-700 nm). An impressive six log units of reduction in viable cells was observed for methicillin-resistant \textit{S. aureus} (Gram-positive), 5-6 log units for \textit{A. baumannii} and MDRAB (Gram-negative), and ~2.5 log units for \textit{P. aeruginosa} (Gram-negative) when using a 20 \(\mu\)M suspension of CNC-Por (1). When viewed alongside our previous study which demonstrated that 1 was able to inactivate \textit{S. aureus} (MSSA) by 5-6 log units reduction of viable cells, \textit{M. smegmatis} by 3-4 log units, and \textit{E. coli} by 1-2 log units, it is possible to conclude that CNC-Por (1) is able to mediate the photodynamic inactivation of bacteria across order, class, and phylum. Confocal microscopy demonstrated that the mode of action for CNC-Por (1) likely does not proceed through a PS-binding or uptake mechanism. Rather, the cytotoxic species (e.g., \(1^1O_2\)) generated by the photodynamic process likely diffuses over short distances to ultimately damage the bacterium, leading to cell death. This contrasts with Zn-EpPor (3), which as a solution-based photosensitizer was shown to directly interact...
with *A. baumannii*, and provides insight into the mode of action of soluble photosensitizers vs. photobactericidal materials. Given the potential shown by this one cellulose-porphyrin conjugate to rapidly photoinactivate a wide range of bacteria with high efficiency, future efforts will focus on the systematic modification of the porphyrin (e.g., charge, hydrophobicity, meso-substituents) to create a library of PS-cellulose nanocrystalline systems that will allow for elucidating the fundamental interactions that govern PDI of bacteria through these cellulose derivatives, while at the same time leading to the development of potent photobactericidal materials with broad spectrum antimicrobial activity to combat the growing threat to human health posed by the transmission of pathogenic bacteria.

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### 3.8 References


3.9 Supplementary Materials

**Solid State UV-visible Spectroscopic Characterization of CNC-Por (1) and Zn-EpPor (3).**

Solid-state UV-visible absorption spectra were collected at room temperature with a Shimadzu UV-3600 spectrophotometer employing the Shimadzu UV-Probe software package. Samples of CNC-Por (1) and Zn-EpPor (3) were placed directly on separate barium sulfate plates. The reflectance data for each was recorded from 200-1800 nm, and the Kubelka-Munk conversion was applied to the raw data to correct for distortions. The corrected transmission data were converted to absorbance spectra for visualization in Figure 3.S1.
Figure 3.S1. Solid state UV-visible Spectra of A) CNC-Por (1) and B) Zn-EpPor (3).
Chapter 4

The Synthesis, Characterization, and Antimicrobial Efficacy of Photomicrobicidal Sheets

Bradley L. Carpenter ¹, Frank Scholle ², Aaron J. Francis ¹, Hasan Sadeghifar ³,⁴, Jonathan Boltersdorf ¹, Walter W. Weare ¹, Dimitris S. Argyropoulos ¹,³,⁵, Paul A. Maggard ¹, Reza A. Ghiladi *¹

¹ Department of Chemistry, North Carolina State University, Raleigh, North Carolina, 27695-8204
² Department of Microbiology, North Carolina State University, Raleigh, North Carolina, 27695-7615
³ Department of Forest Biomaterials, North Carolina State University, Raleigh, North Carolina, 27695-8005
⁴ Department of Wood and Paper Science, Sari Branch, Islamic Azad University, P.O. Box 48161-19318, Sari, Iran
⁵ Department of Chemistry, University of Helsinki, Helsinki Finland

This manuscript is currently being prepared for submission. Bradley L. Carpenter contributed the antibacterial studies, the porphyrin synthesis, and the characterizations. Frank Scholle contributed the antiviral studies. Aaron J. Francis contributed the BODIPY synthesis. Hasan Sadeghifar contributed the fiber and porphyrin preparations, as well as the cellulose fiber FTIR experiments. Jonathan Boltersdorf assisted with UV-vis DRS experiments.
4.1 Abstract

Toward our goal of scalable, antimicrobial materials based upon photodynamic inactivation, photosensitizer-conjugated cellulose fibers were prepared using porphyrin and BODIPY photosensitizers, and subsequently pressed into paper sheets. The photosensitizer-conjugated papers were characterized by infrared, UV-vis diffuse reflectance, and inductively coupled plasma optical emission spectroscopies, as well as thermal gravimetric analysis. Antibacterial efficacy was evaluated against *Staphylococcus aureus* (ATCC-2913), vancomycin-resistant *Enterococcus faecium* (ATCC-2320), *Acinetobacter baumannii* (ATCC-1605), *Pseudomonas aeruginosa* (ATCC-9027) and *Klebsiella pneumoniae* (ATCC-2146). Our best results were achieved with a cationic porphyrin-paper conjugate, *Por*(+)-paper, with inactivation of all bacterial strains studied by ~99.99+% (4 log units) regardless of taxonomic classification (30 min illumination, 65±5 mW/cm², 400-700 nm). *Por*(+)-paper also inactivated dengue-1 virus (>99.995%), influenza A (~99.5%), and human adenovirus-5 (~99%). These results demonstrate the potential for scalable cellulose scaffolds as anti-infective or self-sterilizing materials against both bacteria and viruses when employing a photodynamic inactivation mode of action.

4.2 Introduction

As microbial drug-resistance has increased in prevalence, previously treatable infections have re-emerged as serious and life-threatening healthcare challenges. Though typically not a direct threat to healthy individuals, drug-resistant nosocomial infections affect 1 out of every 20 hospital patients in the United States alone,¹ contributing to the 100,000 deaths attributed to hospital acquired infections (HAIs) annually.¹ While many strategies to combat HAIs have been proposed, including new drug-discovery efforts, alternative treatment modalities, conservative use of antibiotics, and improvements to the FDA approval process,² preventative measures remain a crucial strategy in reducing the number of deaths due to HAIs. In light of this, a renewed focus on sterilization methods, and in particular self-sterilizing materials, has emerged as one such preventative strategy, since it has been found that strains
of the most common bacterial pathogens can survive on hospital environment surfaces, such as bed linens, drapes, and counters, for many months prior to transmission. Materials which are actively antimicrobial could prevent the transmission of pathogenic microorganisms autonomously, without the potential for human error leading to infections. These materials have the potential to contribute to a self-sterilizing hospital environment in a number of applications, including antimicrobial bedding, hospital drapes, patient gowns, staff uniforms, and countertops. Such applications, however, currently remain only ‘potential’ as our understanding of these materials, including their physical properties, scalability, scope of utilization, and efficacy as anti-infectives, is still developing.

There has been an increased interest in employing antimicrobial photodynamic inactivation (aPDI) as the basis for pathogen eradication given its advantages over other sterilization options. These include: i) the photosensitizers, visible light excitation, and material scaffolds themselves are non-toxic, and are therefore not expected to have an adverse effect on human health; ii) their antimicrobial action is mediated by singlet oxygen, which has several benefits including a short lifetime before it decays back to environmentally benign ground state (triplet) oxygen, the ability to inactivate bacteria that are not in direct contact with the material, as well as potentially broad antimicrobial efficacy against bacteria, yeast, viruses, and parasites; and iii) as singlet oxygen causes nonspecific damage, it is thought that microbes will be unable to develop resistance to this mode of action. Additionally, many photosensitizers are amenable for conjugation to synthetic and biopolymer based scaffolds, and a number of synthetic strategies have been reported, including the use of esterification reactions, the Cu(I)-catalyzed Huisgen-Meldal-Sharpless 1,3-dipolar cycloaddition reaction, amine- or amide-linker forming conjugations, as well as several other strategies. Early investigations into aPDI materials were initially inhibited by the belief that, to be effective, photosensitizers needed to either be embedded in the cellular envelope or internalized into the bacteria. This belief has now been thoroughly disproved as advances in our scientific understanding of the mechanisms of antimicrobial photodynamic inactivation of bacteria have advanced.

Toward our goal of scalable, self-sterilizing materials based upon aPDI, we present
here the preparation of photosensitizer-conjugated cellulose fibers that were subsequently pressed into paper sheets. Cellulose materials have many desirable physical characteristics, including inherent biocompatibility, and provide an inexpensive starting material that is easily scalable. Our early work employing cellulose nanocrystals modified with a cationic porphyrin, termed CNC-Por, validated this approach given the excellent antibacterial efficacy observed for that material. However, given the limitations of cellulose nanocrystals (e.g., limited scalability, additional preparation requirements), we have instead chosen to work here with cellulose fibers. The corresponding antimicrobial paper sheets were prepared with a diverse set of photosensitizers, including cationic, anionic, and neutral porphyrins as well as neutral BODIPY based photosensitizers, and characterized using FTIR, UV-vis DRS and ICP-OES spectroscopies as well as TGA. The photosensitizer-conjugated paper sheets were subsequently evaluated for their antibacterial efficacy against a set of taxonomically diverse bacteria, all of which were members of the so-called ‘ESKAPE’ pathogens, known to be the most common causes of hospital acquired infections. We also studied our most effective PS-paper against both enveloped and non-enveloped viruses to determine its antiviral efficacy. Our results demonstrate the potential for aPDI materials based upon scalable cellulose scaffolds as anti-infective materials against both bacteria and viruses.

4.3 Materials and Methods

Materials. All specialty chemicals for photosensitizer synthesis were purchased either from VWR or Sigma Aldrich, and were used without further purification. Salts for the preparation of phosphate buffered saline solution were purchased from Fisher Scientific. Nutrient Broth #234000 and Heart Brain Broth #237500 were purchased from BD Difco, Miller LB broth was purchased from EMD Chemicals, and Tryptic Soy Broth was purchased from Teknova. Whatman grade 1 filter paper was used to harvest cellulose fibers. Fisherbrand filter paper qualitative P8 was used for all gravity filtrations. UV-visible spectra were recorded on a Varian Cary 50 Bio spectrophotometer, and a Genesys 10 UV scanning spectrophotometer (Thermo Electron Corp.) was used for single wavelength measurements. ¹H- and ¹³C-NMR
spectra were acquired on Varian 300 or 400 MHz (\(^{13}\)C: 75 or 100 MHz) NMR spectrometers with chemical shifts reported as \(\delta\) (ppm) values calibrated to natural abundance deuterium solvent peaks. Matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS) was performed with an AB Sciex TOF/TOF 5800. A Perkin Elmer 2400 CHNS analyzer was used for CHN elemental analysis. FTIR (Fourier transform infrared) spectra of unconjugated porphyrins were acquired on a Jasco FT/IR-4100 spectrometer while spectra of photosensitizer-fiber conjugates were acquired on a Thermo Nicolet NEXUS 670 FT-IR spectrophotometer as KBr pellets. PS-fiber conjugate spectra in the range of 4000-650 cm\(^{-1}\) were obtained with a resolution of 4 cm\(^{-1}\) by accumulating 64 scans. UV-vis diffuse reflectance (UV-vis DRS) spectra were collected on a Shimadzu UV-3600 spectrophotometer equipped with an integrating sphere utilizing the wavelength range of 200-1500 nm. Thermal gravimetric analysis (TGA) was carried out on a TA instrument TGAQ500.

**Cellulose Fiber Preparation.** Mother fibers were prepared from Whatman #1 filter paper. The filter paper was soaked with water for 2 hours to open the fibers, then washed 3 times with acetone to clean and remove impurities. The fibers were subsequently dried at room temperature for 72 h and fluffed using a coffee grinder. Azido fibers and photosensitizer conjugated fibers were prepared with the same methodology as reported previously.\(^{5b,10}\) using the cellulose fibers obtained here *in lieu* of the cellulose nanocrystals used in that earlier work.

Fibers were pressed into paper sheets using the Tappi T205 sp-02 standard method.\(^{11}\) The appropriate fibers were suspended in water to create a 1% (w:v) fiber suspension (1 g fibers/100 mL H\(_2\)O). The fibers were disintegrated using a TMI Durant Pulp Disintegrator at 3,000 rpm for 10 min. The suspension was then transferred into a cylindrical British sheet making machine (173 mm diameter) and 3 additional liters of water were added. The water was then drained leaving a wet sheet. The sheet was further dried by pressing with blotting paper before bringing to dryness on a hot plate.
Scheme 4.1. General route for the synthesis of PS-paper conjugates.

Photosensitizer Synthesis

5-(4-Ethynylphenyl)-10,15,20-tris(N-methylpyridinium) porphyrin Zn(II) (Por$^{(+)}$) (I). The cationic water-soluble porphyrin Por$^{(+)}$ (I) was synthesized as previously reported by Feese et al.$^{5b}$
1,3,5,7-Tetramethyl-8-(4-propargyloxyphenyl)-4,4-difloroboradiaza-s-indacene (Bdy\(^{2H}\)) (2). The neutral boron-dipyrrromethene compound Bdy\(^{2H}\) (2) was synthesized according to Erbas et al.\(^{12}\) with slight modifications as follows: 4-(prop-2-ynyloxy)benzaldehyde (2.0 g, 12.4 mmol) was dissolved in methylene chloride (745 mL) and absolute ethanol (55 mL). The solution was sparged (N\(_2\), 15 min) followed by addition of 2,4-dimethylpyrrole (2.6 mL, 25.0 mmol) and 1 drop TFA. The solution was stirred at room temperature for 24 h to yield a homogeneous red-amber mixture, after which chloranil (3.07 g, 12.4 mmol) was added and the solution turned black. The solution was stirred an additional 2 h, followed by addition of Hunig's base (13.0 mL, 75 mmol). The solution was stirred 20 min, then BF\(_3\)-etherate (12.5 mL, 100 mmol) was added and it was stirred for 1 h. The crude mixture was filtered through a pad of basic alumina and silica, then further purified by flash chromatography (silica, CH\(_2\)Cl\(_2\), 7 cm i.d. \(\times\) 25 cm), affording a 17% yield (800 mg, 2.12 mmol) of 2 as an orange powder. Characterization (\(^1\)H-NMR, UV-vis) matched that reported in the literature.\(^{12-13}\)
2,6-Diido-1,3,5,7-tetramethyl-8-(4-propargyloxyphenyl)-4,4-difloroboradiazas-indacene (Bdy\textsuperscript{2I}) (3). The diido boron-dipyrromethene Bdy\textsuperscript{2I} (3) was prepared as described in Erbas et al.\textsuperscript{12}
5-[4-(Trimethylsilyl)ethynylphenyl]-10,15,20-tris(4-carbomethoxyphenyl) porphyrin (4). This compound was synthesized using a procedure analogous to that reported by Wang et al.\textsuperscript{13} as follows: To a 1 L three-neck flask was added 500 mL of dry chloroform (dispensed from a solvent purifier), 1.2 g (7.5 mmol) of methyl 4-formylbenzoate, 500 mg (2.5 mmol) of 4-[(trimethylsilyl)ethynyl]benzaldehyde, and 0.7 mL (10 mmol) freshly distilled pyrrole. The solution was sparged (Ar, 10 min), followed by the addition of 350 µL BF\textsubscript{3}∙Et\textsubscript{2}O (ca. 48% BF\textsubscript{3}). The solution was stirred in the dark under an argon atmosphere for 2 h, after which 1.1 g p-chloranil was added, and the solution was stirred overnight open to air. The solvent was evaporated, and the residue was redissolved in a minimum volume of CH\textsubscript{2}Cl\textsubscript{2} before being run through a silica plug (CH\textsubscript{2}Cl\textsubscript{2}, 10 cm o.d. × 6 cm) to remove the tar byproduct. Purification was completed using flash column chromatography (silica, CH\textsubscript{2}Cl\textsubscript{2}, 5.5 cm o.d. × 18 cm, \(R_f = 0.6\)). Compound 4 precipitated to form brown microcrystals with the addition of methanol and evaporation of the CH\textsubscript{2}Cl\textsubscript{2} yielding ~220 mg (250 µmol, 10%). Characterization (\textsuperscript{1}H-NMR, MALDI-TOF-MS) matched that reported in the literature:\textsuperscript{13} \textsuperscript{1}H-NMR (300 MHz, CDCl\textsubscript{3}): \(\delta\) 8.81 (m, 8H), 8.45 (d, 6H), 8.29 (d, 6H), 8.15 (d, 2H), 7.88 (d, 2H), 4.12 (s, 9H), 0.377 (s, 9H), -2.82 (s, 2H); MALDI-TOF-MS \((m/z)\): obsd 886.34, calcd 885.32 \([(M+H)^+\], M = C\textsubscript{55}H\textsubscript{44}N\textsubscript{4}O\textsubscript{6}Si].

\[\text{H}\]
5-[4-(Trimethylsilyl)ethynyl-phenyl]-10,15,20-tris-(4-carbomethoxyphenyl)porphyrin Zn(II) (5). To a 500 mL three-neck flask was dissolved 500 mg (4) (565 µmol) in 250 mL of THF and subsequently deoxygenated (Ar, 10 min). To this was added 989 mg (4.5 mmol) zinc (II) acetate dissolved in the minimal requisite volume of methanol (~1 mL). The solution was refluxed for 2 hours under argon. MALDI-TOF-MS was used to confirm reaction completion, after which the product was precipitated by the slow evaporation of THF upon rotary evaporation and addition of methanol. The precipitate was collected using centrifugation, washed 5 × 10 mL MeOH to remove excess zinc (II) acetate, and was then dried in vacuo to afford 5 as purple microcrystals. $^1$H-NMR (300 MHz, CDCl$_3$): δ 8.91 (m, 8H), 8.42 (d, 6H), 8.157 (d, 2H), 7.88 (d, 2H), 4.09 (s, 9H), 0.38 (s, 9H); MALDI-TOF-MS (m/z): obsd 946.20, calcd 947.24 [(M+H)$^+$, M = C$_{55}$H$_{42}$N$_4$O$_6$SiZn].
5-(4-Ethynylphenyl)-10,15,20-tris(4-carboxyphenyl) porphyrin Zn(II) (Por\(^{4+}\)) (6).

To a 250 mL round bottom flask was added 500 mg (527 µmol) (5) dissolved in 100 mL 1:1 THF:NaOH\(_{aq}\) (1 M). The reaction was stirred at room temperature for 3 d, after which the THF solvent was removed \textit{in vacuo} leading to a partial precipitation of the product. The resulting suspension was diluted 1:10 into deionized water, redissolving the precipitate, and the pH was lowered by the careful addition of 1 M HCl with stirring until the product precipitated completely. The crude product was collected by centrifugation, and the supernatant was further decanted through filter paper to collect product still suspended in solution. To remove excess HCl, the precipitate was washed several times by being resuspended in ~25 mL deionized water and centrifuged until the supernatant began to show color. The precipitate was then dissolved in MeOH and concentrated \textit{in vacuo} resulting in 6 as a purple powder in 90+ % yield (>400 mg, 480 mmol). \(^1\)H-NMR (300 MHz, CD\(_3\)OD): \(\delta\) 8.84 (m, 8H), 8.43 (d, 6H), 8.31 (d, 6H), 8.186 (d, 2H), 7.87 (d, 2H), 3.73 (s, 1H); MALDI-TOF-MS (m/z) obsd 832.15, calcd 833.15 [(M+H)+, \(M = C_{49}H_{28}N_4O_6Zn\)]; \(\lambda_{abs}\) (DMSO): 431 (Soret), 523, 562, 602 nm; FTIR (KBr): 1602, 1685, 2344, 2846, 2918, 3437 cm\(^{-1}\).
**5-(4-Ethynlyphenyl)-10,15,20-tris(4-carboxyphenyl) porphyrin (7).** To a 250 mL round bottom flask was added 500 mg (565 mmol) (4) dissolved in 100 mL 1:1 THF:NaOH$_{aq}$ (1 M). Solution was stirred at room temperature for three days. Upon removal of the THF *in vacuo* some of the product was seen to precipitate. The suspension was diluted 1:10 into deionized water, redissolving the precipitate. While stirring, the pH was lowered by the careful addition of 1 M HCl until the product precipitated completely. The product was collected by centrifugation followed by gravity filtration. The collected brown precipitate was subsequently washed several times with deionized water until the eluent began to show coloring. The precipitate was dissolved in MeOH and concentrated *in vacuo* to give 7 as a brown powder +90% yield (>390 mg, 506 µmol). $^1$H-NMR (300 MHz, CD$_3$OD): $\delta$ 8.85 (s, 8H), 8.41 (d, 6H), 8.28 (d, 6H), 8.18 (d, 2H), 7.86 (d, 2H), 3.12 (s, 1H); MALDI-TOF-MS ($m/z$): obsd 770.90, calcd 771.24 [(M+H)$^+$, M = C$_{49}$H$_{30}$N$_4$O$_6$]; $\lambda_{abs}$ (DMSO): 421 (Soret), 514, 551, 590, 646 nm; FTIR (KBr): 1635, 1686, 2343, 2849, 2919, 3431 cm$^{-1}$. 
5-(4-Ethynylphenyl)-10,15,20-tris[4-(N-dodecylcarbamoyl)phenyl] porphyrin (8). To a 100 mL round bottom flask was added 300 mg (390 µmol) (7), 90 mL chloroform containing 3% TEA, 546 mg (2.95 mmol) dodecylamine, 468 mg (2.27 mmol) dicyclohexylcarbodiimide, and 308 mg (2.01 mmol) hydroxybenzotriazole monohydrate. The mixture was stirred at room temperature for 2 days in the dark. The reaction mixture was gravity filtered. The filtrate was collected, and the solvent was removed in vacuo at room temperature. The residue was dissolved in a minimal amount of chloroform with 3% TEA and the product was separated via flash chromatography (silica, 97:3 CHCl₃:TEA, 5.5 cm o.d. × 35 cm, Rₜ = 0.4). The product peak was collected and recrystallized with the removal of CHCl₃ and addition of MeOH to give 8 as brown microcrystals in 55-67% yield (273-332 mg, 214-261 µmol). H-NMR (300 MHz, CDCl₃): δ 8.80 (m, 8H), 8.26 (t, 6H), 8.16 (m, 8H), 7.89 (d, 2H), 6.437 (m, 3H), 3.605 (m, 6H), 3.33 (s, 1H), 1.77 (m, 6H), 1.2-1.6 (m, 63H observed vs. 54H expected due to overlap with H₂O), 0.88 (m, 9H), -2.83 (s, 2H). MALDI-TOF-MS (m/z): obsd 1274.15, calcd 1272.85 [(M+H)+, M = C₈₅H₁₀₅N₇O₃]; λₗₘₚ(CHCl₃): 421 (Soret), 451, 516, 551, 590, 650 nm.
5-(4-Ethynylphenyl)-10,15,20-tris[4-(N-dodecylcarbamoyl)phenyl] porphyrin Zn(II) (Por₀) (9). To a 500 mL 3-neck flask was added 500 mg (393 µmol) (8) in 250 mL THF, the solution was deoxygenated (Ar, 10 min), and 989 mg (4.5 mmol) zinc (II) acetate dissolved in the minimally requisite volume of methanol (~1 mL) was subsequently added to the solution. The reaction mixture was refluxed for 2 h under argon. MALDI-TOF-MS was used to confirm reaction had gone to completion, after which the product was precipitated by the slow evaporation of THF upon rotary evaporation with addition of methanol. The precipitate was collected using centrifugation, washed 5 × 10 mL MeOH to remove excess zinc (II) acetate, and then dried in vacuo affording 9 as purple microcrystals (~500 mg, 374 µmol). \(^1\)H-NMR (300 MHz, DMSO): \(\delta\) 8.78 (m, 8H+3H), 8.25 (s, 12H), 8.19 (d, 2H), 7.90 (d, 2H), 4.42 (s, 1H), 3.40 (m, 6H+H₂O), 1.39 (m, 6H), 1.27 (m, 54H), 0.82 (m, 9H); \(^{13}\)C-NMR (500 MHz, DMSO): \(\delta\) 167.03, 149.93, 149.90, 146.11, 144.12, 135.22, 134.89, 134.64, 132.60, 130.86, 126.31, 121.87, 120.65, 120.48, 84.44, 82.71, 67.87, 55.79, 32.19, 30.07, 29.97, 29.93, 29.73, 29.62, 27.44, 22.98, 14.84; MALDI-TOF-MS (m/z): obsd 1334.78, calcd 1334.76 [(M+H)+, \(M = C_{85}H_{103}N_7O_3Zn\)]; \(\lambda_{abs}\) (DMSO): 431 (Soret), 523, 561, 601 nm; FTIR: 1602, 1685, 2344, 2846, 2918, 3437 cm\(^{-1}\).
Por\(^{(+)}\)-paper. This material was prepared as described above with 700 mg mother fibers and 500 mg (667 µmol) Por\(^{(+)}\) (1) as the photosensitizer substrate eventually resulting in an ~1 g sheet of Por\(^{(+)}\)-paper ~16 cm in diameter. UV-vis DRS: 453 (Soret), 572, 615 nm; CHN Elemental: 39.07% C, 6.01% H, 1.07% N.

Por\(^{(-)}\)-paper. This material was prepared as described above with 700 mg of mother fibers and 500 mg (602 µmol) Por\(^{(-)}\) (6) as the photosensitizer substrate eventually resulting in a ~1 g sheet of Por\(^{(-)}\)-paper ~16 cm in diameter. UV-vis DRS: 431 (Soret), 562, 605 nm; CHN Elemental: 40.76% C, 7.05% H, 0.47% N.
Por\(^{(0)}\)-paper. This material was prepared as described above (substituting DMF with 1:1 DMF:CH\(_2\)Cl\(_2\) to solvate the photosensitizer) with 700 mg of mother fibers and 500 mg (374 µmol) Por\(^{(0)}\) (9) as the photosensitizer substrate to give an ~1 g sheet of Por\(^{(0)}\)-paper ~16 cm in diameter. UV-vis DRS: 431 (Soret), 561, 602 nm; CHN Elemental: 41.13% C, 4.11% H, 0.17% N.
**Bdy**(2H)-paper. This material was prepared as described above (substituting DMF with 1:1 DMF:CH₂Cl₂ to solvate the photosensitizer) with 700 mg mother fibers and 400 mg (Bdy**(2H)** 1.06 mmol) (2) as the photosensitizer substrate to give an ~1 g sheet of **Bdy**(2H)-paper ~16 cm in diameter.
**Bdy**(21)-paper. This material was prepared as described above (substituting DMF with 1:1 DMF:CH$_2$Cl$_2$ to solvate the photosensitizer) with 700 mg mother fibers and 500 mg (790 µmol) Bdy**(21)** (3) as the photosensitizer substrate to give ~1 g sheet of **Bdy**(21)-paper ~16 cm in diameter. UV-vis DRS: 539 (Soret), 395 nm; CHN Elemental: 39.54% C, 6.91% H, 0.61% N.

**Porphyrin loading onto paper sheets.** The extent of porphyrin loading onto the fibers was determined using inductively coupled plasma-optical emission spectroscopy (ICP-OES). Samples were prepared by placing 1 custom hole-punch of each paper sample (~ 1 cm diameter) of known mass (typically between 15 and 20 mg) into a 10 mL volumetric flask and bringing the volume to 10 mL by addition of 1 M HCl. The mixture was stirred vigorously at 60 °C for 2 h and then filtered prior to Zn analysis on a Perkin Elmer Model 8000 Dual View ICP-OES spectrometer.

**Cell Culturing.** All bacterial strains were grown in 5 mL cultures which were incubated on an orbital shaker at 37 °C. The growth conditions were as follows: *Acinetobacter baumannii* (ATCC-19606) was grown in Miller-LB media without antibiotics; multi-drug resistant *Acinetobacter baumannii* (ATCC-1605) was grown in Miller-LB media with 5 µg/mL tetracycline; methicillin-susceptible *Staphylococcus aureus* ATCC-2913 was grown in tryptic
soy broth media without antibiotics; methicillin-resistant *Staphylococcus aureus* (ATCC-44) was grown in tryptic soy broth media with 5 µg/mL tetracycline; *Pseudomonas aeruginosa* (ATCC-9027) was grown in BD Difco Nutrient Broth #234000; *Klebsiella pneumoniae* (ATCC-2146) was grown in BD Difco Nutrient Broth #234000 with 100 µg/mL ampicillin. The vancomycin-resistant strain of *Enterococcus faecium* (ATCC-2320) was grown in BD Difco Bacto Brain Heart Infusion #237500 with 50 µg/mL ampicillin. Bacteria were grown to a concentration of 1–4 × 10^8 CFU/mL (determined spectrophotometrically from growth curves using a Genesys 10 UV scanning spectrophotometer) prior to being pelleted by centrifugation (10 min, ~3700 g). Once pelleted, the supernatant was decanted, and the cells were resuspended in 5 mL of PBS (170 mM NaCl, 3.4 mM KCl, 10.0 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.2).

**Viral Propagation.** The Dengue 1 virus was propagated for 168 h on C6/36 mosquito cells in L15 medium supplemented with 10% FBS (fetal bovine serum), antibiotics, and 10% tryptose phosphate broth before the virus was harvested. Human adenovirus-5 (HAd-5) was propagated for 96 h on the human lung carcinoma cell line A549 in DMEM (Dubelco’s Modified Eagle’s Medium) with 10% FBS and antibiotics before the virus was harvested. Influenza A (PR8/34) was propagated for 48 h on Madine-Darby canine kidney cells (MDCK) in DMEM with 0.2% BSA (bovine serum albumin), 25 mM HEPES buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), antibiotics, and 2 µg/mL TPCK (Tosyl phenylalanyl chloromethyl ketone)-treated trypsin before the virus was harvested. Antibiotics for all viral media were brought to a final concentration of 100 IU/mL Penicillin, 100 IU/mL Streptomycin, 0.25 µg/mL.

**Antimicrobial Photodynamic Inactivation Assay (aPDI).** All antimicrobial photodynamic inactivation studies of the papers were carried out using a non-coherent light source, PDT light model LC122 (LumaCare, USA) and an Orion power meter (Orphir Optronics Ltd, Israel) to measure the fluence rate. All experiments were carried out in triplicate at a minimum (unless noted otherwise), and statistical significance was assessed via a two tailed, unpaired Student’s t-test.

**Bacteria:** For each antibacterial PDI trial, duplicate 24-well plates (Nunc, flat bottom)
were prepared by placing into adjacent wells one circular sheet of Whatman grade 1 filter paper (as a PS-free control) and one circular sheet of PS modified paper. Each sheet was cut to precisely fit the well bottom (~1 cm dia.) using a custom hole-punch. A 100 µL aliquot of a single 5 mL bacterial culture was then added to each of the four wells. Once inoculated, one of the well plates was kept in the absence of light as a dark control, and the other was illuminated for 30 minutes at 65±5 mW/cm² with non-coherent visible light (400-700 nm). Following illumination, 1 mL of sterile PBS was added to each well in both the illuminated and the dark control plate, and the bacteria were resuspended with vigorous stirring and vortexing. Each well was 1:10 serially diluted (40 µL into 360 µL aliquots of PBS) five times and 10 µL of each dilution as well as 10 µL from the undiluted wells were plated in columns on gridded six column square agar plates made with their respective growth media (without antibiotics). The plates were incubated overnight in the dark at 37 °C. The survival rate was determined by the ratio of CFU/mL of the illuminated sample versus that of the identical paper from the dark control. The minimum detection limit was 100 CFU/well (based on the plated 10 µL aliquot from the 1 mL undiluted well). Variations in the number of delivered CFUs (1–4 × 10⁷ CFUs from the broth culture at a concentration of 1–4 × 10⁸ CFU/mL) resulted in a detection limit range of 0.01-0.001% survival.

Viruses: Antiviral photodynamic inactivation studies were carried out with a methodology similar to that of the antibacterial studies. Three sheets of both Whatman grade 1 filter paper (PS free control) and Por⁺-paper were cut using a standard hole-punch (5 mm) to closely fit the well bottom of a 96-well plate (Olympus). 25 µL of the appropriate virus was added to the wells with paper sheets, as well as three without (no paper control) and the plate was subsequently illuminated for 30 minutes at 65±5 mW/cm² with non-coherent visible light (400-700 nm). Another 96 well plate, prepared identically to the first, was kept in the absence of light as a dark control. Following illumination, 100 µL of MEM (Eagle’s Modified Essential Medium) with 1% FBS, 10 mM HEPES, and 1% antibiotics were added to each well in both the illuminated plate and dark control plate. The wells were gently vortexed with a micropipettor to facilitate removal of the viruses from the paper sheets, and each well was 1:10
serially diluted (15 μL into 135 μL aliquots of MEM) six times. To gauge the viral concentration in each of the wells, the viruses were titered on the appropriate cell lines: the Vero E6 cell line was used for titering the dengue 1 virus, the A549 cell line for human adenovirus-5, and the MDCK cell line for the influenza A virus. Each of these target cell lines were grown to confluence in 24 well plates (Falcon, flat bottom). The confluent cells were then infected with 150 μL of the serial dilutions. After one hour of incubation 800 μL of a semisolid overlay (1% tragacanth gum in MEM) was added and the plates were incubated for 24 h (influenza A/MDCK) or 72 h (human adenovirus-5/A549 and dengue-1/Vero E6). For the influenza A virus and MDCK cell line, 2 μg/mL of TPCK-treated trypsin was also added to the semisolid overlay prior to incubation. Each of the wells was fixed with 50:50 acetone/methanol, blocked with 1% normal horse serum for 10 min, and incubated with primary antibodies specific to the viruses: E-protein specific antibody D1-4G2 was used for the dengue 1 virus, adenovirus-2/5 E1A (A-3) antibody (Santa Cruz Biotech.) for HAd-5, and an HA-specific antibody (C102; Thermo Scientific) for the influenza A virus. Immunofoci were visualized after incubation with horseradish peroxidase conjugated anti-mouse IgG and staining using the Vectastain ABC kit specific to mouse IgG (Vector Laboratories). Foci in wells containing 20-70 foci (depending on the virus) were counted for the calculation of focus-forming units (ffu). While the concentration detection limit was 50 ffu/mL for each virus, due to differences in starting viral concentration, the detection limit for % activity varied between viruses.

4.4 Results

Synthesis. Covalent attachment of alkyne-bearing photosensitizers to azide-functionalized cellulose fibers was achieved by the Cu(I)-catalyzed Huisgen-Meldal-Sharpless 1,3-dipolar cycloaddition reaction. Compounds (1), (2), and (3) were prepared per literature precedent. The previously unreported porphyrins Por\(^{1-}\) (6) and Por\(^{0}\) (9) were prepared in 3 and 4 steps, respectively, starting from a statistical condensation that was used to prepare their common precursor 4 (see Supporting Information). Briefly, the byproducts, including
porphyrin isomers and linear pyrrolic polymers (tar), were chromatically separated from the desired free-base porphyrin 4. Metallation with zinc acetate, followed by the removal of protecting groups with aqueous NaOH, afforded Por(+) (6). Alternatively, 4 underwent deprotection to yield the free base porphyrin, the resulting carboxylic acids were then activated through esterification, and substitution with dodecylamine followed by metalation with zinc acetate as the final step yielded Por(0) (9).
Scheme 4.2. The synthesis of $\text{Por}^{(6)}$ and $\text{Por}^{(9)}$. i: $\text{BF}_3\cdot\text{EtO}_2$, CHCl$_3$, rt, 2 h. 2. TCQ, rt, 12 h. ii: Zn (II) acetate, THF, reflux, 2 h. iii: THF:NaOH$_{aq}$ (1 M), rt, 3 days. iv: HOBt, DCC, dodecylamine, CHCl$_3$ (3% TEA), rt, 48 h.
Azide-functionalized cellulose fibers (Az-fibers) were prepared as previously reported, with the exception that where cellulose nanocrystals were used previously, cellulose fibers were used in these reactions. Conditions for the Cu(I)-catalyzed Huisgen-Meldal-Sharpless 1,3-dipolar cycloaddition reaction were followed from the literature for the reaction of Por\(^{(+)}\) (1) and Por\(^{(-)}\) (6) with Az-fibers in DMF, yielding Por\(^{(+)}\)-paper and Por\(^{(-)}\)-paper, respectively, which were pressed directly into paper sheets. To allow for the solubilization of the more nonpolar photosensitizers [Por\(^{(0)}\) (9), Bdy\(^{(2H)}\) (2) and Bdy\(^{(2I)}\) (3)], a 1:1 CH\(_2\)Cl\(_2\):DMF reaction solvent was utilized. However, once pressed into paper sheets, it was found that PBS and deionized water beaded on their surface, preventing the bacteria from interacting with the material. To allow the PBS bacterial culture access to these hydrophobic fibers, the photosensitizer modified fibers were mixed (doped) with either 25 wt\% (Bdy\(^{(2H)}\) and Bdy\(^{(2I)}\)) or 50 wt\% (Por\(^{(0)}\)) unmodified cellulose mother fibers. This process was found to be effective at allowing PBS to absorb into the sheets, and thereby allow bacteria better proximity to the photosensitizers. It was observed that this process increased the error for the associated aPDT studies of these papers \((\textit{vide infra})\), this is likely due to the heterogeneity in photosensitizer distribution arising from the incomplete mixing of the PS-conjugated and mother fibers in the resulting paper.

**Characterization.**

\textbf{FTIR characterization of photosensitizer modified fibers.} FTIR characterization was used to assess the synthetic modifications of the mother fibers, including both the nucleophilic addition of azide (yielding Az-fibers) and the conjugation of photosensitizers (yielding PS-fibers). For accurate comparison of these products, the transmission spectra were normalized to the 1030 cm\(^{-1}\) band. The Az-fiber spectra (Figure 4.1, panels A and B, black trace) show a clear azide band at 2113 cm\(^{-1}\), confirming the presence of the azide-modified cellulose fibers. The amount of azide loading, as determined by the area of this band in the normalized spectra, was found to be lower in Az-fibers than in the previously studied cellulose nanocrystals, and can be rationalized given the lower percent surface area of the fibers.
relative to the cellulose nanocrystals. Since the Huisgen-Meldal-Sharpless 1,3-dipolar cycloaddition reaction converts azide functional groups to triazole linkers, the addition of photosensitizers to the Az-fibers could be measured as a % reduction in the azide band area. The Por\(^{(+)}\)-fiber spectrum (Fig. 4.1A, red trace) shows a decline in the azide band area (~30%), confirming covalent attachment of the photosensitizer, albeit at a lower efficiency than that observed for Por\(^{(+)}\) addition to cellulose nanocrystals (72%). Interestingly, the spectrum of the Por\(^{(-)}\)-fibers (Figure 4.1A, green trace) showed a greater extent of photosensitizer conjugation than did Por\(^{(+)}\)-fibers, with the azide band significantly reduced in intensity. This higher loading may be in part due to lower charge density present on the Por\(^{(-)}\) \( \text{porphyrin} \) (as the porphyrin was conjugated to the Az-fibers in triprotic/neutral form, resulting in less charge-charge repulsion than in the Por\(^{(+)}\)-fiber reaction. The Por\(^{0}\)-fiber spectrum (Figure 4.1A, orange trace) also showed no azide peak. However, because FTIR analysis was carried out after doping with 50 wt% mother fibers, the lack of an azide band is more attributable to the dilution with mother fibers (see Elemental Analysis and ICP-OES for further discussion, vide infra) combined with the photosensitizer conjugation. An additional feature seen in the spectrum of the Por\(^{0}\)-fiber is the reduction of the large absorbance at ~3300 cm\(^{-1}\). This may also be a result of the doping process, or it could be due to the lower hygroscopic nature of the neutral Por\(^{0}\)-fiber, causing it to absorb less water from its environment.
Figure 4.1. FTIR spectra of (A) Az-fiber (black), Por\(^{(+)}\)-fiber (red), Por\(^{(0)}\)-fiber (orange), and Por\(^{(-)}\)-fiber (green), and (B) Az-fiber (black), Bdy\(^{(2H)}\) (dark blue), and Bdy\(^{(2I)}\) (light blue).

The FTIR spectra of Bdy\(^{(2H)}\)-fibers (dark blue) and Bdy\(^{(2I)}\)-fibers (light blue) are shown in Figure 4.1B. Only a trace azide band can be observed in the Bdy\(^{(2H)}\)-fiber spectrum, suggesting near complete surface modification. By contrast, Bdy\(^{(2I)}\)-fiber showed lower reactivity in comparison to the dihydro analog, but still exhibited a ~50% reduction in the azide band when compared with the precursor Az-fibers. Overall, both BODIPY compounds were observed to have greater reactivity with Az-fibers than Por\(^{(+)}\) (1), supporting the hypothesis that lower charge-charge repulsion leads to greater surface modification, although the smaller size of the BODIPY photosensitizers may be a factor as well. It should be noted that these spectra were recorded prior to the doping of Bdy\(^{(2H)}\)-fiber and Bdy\(^{(2I)}\)-fiber with mother fibers.

ICP-OES. ICP-OES was used to determine the Zn concentration in the porphyrin-paper materials, which is equivalent to the porphyrin loading if complete metallaition of the
porphyrin is assumed. The Por\(^{(+)}\)-paper was found to have the highest porphyrin loading at 33.2 nmol porphyrin per mg of paper (Table 4.1), in agreement with the FTIR results. The Por\(^{(+)}\)-paper was found to have a loading of 12.4 nmol porphyrin per mg paper. This calculates to ~one-third the amount of porphyrin loading in comparison to Por\(^{(-)}\)-paper, and again closely agrees with the FTIR data. The doped Por\(^{(0)}\)-paper was found to have a loading of 3.54 nmol porphyrin per mg paper, implying that the pre-doped fibers would have ~7 nmol porphyrin per mg paper, still only about half that of the Por\(^{(+)}\)-paper and less than a fourth that of the Por\(^{(-)}\)-paper. This strongly suggests that Por\(^{(0)}\) (9) had a lower efficiency in its reaction with the Az-fibers when compared to the other porphyrin-based photosensitizers. This lower loading could be attributable to the large size of the aliphatic groups leading to steric hindrance with the Az-fibers. ICP-OES was not performed on Bdy\(^{(2H)}\)-paper or Bdy\(^{(2I)}\)-paper since neither photosensitizer possessed Zn or another metal that could be comparably analyzed.

**Table 4.1. ICP-OES Zn analysis of the porphyrin-modified papers.**

<table>
<thead>
<tr>
<th>Material</th>
<th>sample mass (mg)</th>
<th>nmol porphyrin/ sheet</th>
<th>nmol porphyrin/ mg paper</th>
</tr>
</thead>
<tbody>
<tr>
<td>Por(^{(+)})-paper</td>
<td>13.1</td>
<td>168</td>
<td>12.4</td>
</tr>
<tr>
<td>Por(^{(0)})-paper</td>
<td>28.5</td>
<td>101</td>
<td>3.54</td>
</tr>
<tr>
<td>Por(^{(-)})-paper</td>
<td>17.2</td>
<td>570</td>
<td>33.2</td>
</tr>
</tbody>
</table>

**UV-vis Diffuse Reflectance Spectroscopy.** UV-vis diffuse reflectance spectra of Por\(^{(+)}\)-paper [453 (Soret), 572, 615 nm] was found to be shifted bathochromically ~10 nm from the previously reported Por\(^{(+)}\)-CNCs (H\(_2\)O). \(^{5c}\) A bathochromatic shift was also seen going from Por\(^{(+)}\) to Por\(^{(+)}\)-CNCs, likely due to a similar effect as conjugation to the cellulose surface reduced the solvation of the photosensitizer and in UV-vis DRS solvent was entirely removed. Bdy\(^{(2H)}\)-paper [395, 539 nm] showed only minor (<5 nm) shifts in absorbance from its precursor compound. Por\(^{(-)}\)-paper [431 (Soret), 562, 605 nm] and Por\(^{(0)}\)-paper [431 (Soret), 561, 602 nm] showed only minor shifts from the spectra of their precursor compounds Por\(^{(-)}\) (6) and
Por\textsuperscript{(0)} (9). This suggests that no significant changes to the porphyrin or BODIPY precursors, including no transmetallation with the copper catalyst, occurred during the Huisgen-Meldal-Sharpless 1,3-dipolar cycloaddition reaction (other than that of solvation). Metallation with copper would lead to significant changes in the absorption spectrum as noted previously.\textsuperscript{14}

\textbf{Figure 4.2.} UV-vis diffuse reflectance spectroscopy of (A) Por\textsuperscript{(+)}-paper and Bdy\textsuperscript{(2I)}-paper and (B) Por\textsuperscript{(-)}-paper and Por\textsuperscript{(0)}-paper.

\textbf{Thermal Gravimetric Analysis.} Thermal gravimetric analysis was performed on the precursor fibers as well as on Por\textsuperscript{(+)}-paper and Bdy\textsuperscript{(2I)}-paper to gain an understanding of the thermal stability of these materials (Figure 4.3). Apart from a minor initial weight loss that was observed for all materials up to 100 °C (attributable to loss of water), each was found to be stable in excess of 250 °C. The mother fibers (black) proved the most robust to thermal degradation (up to ~330 °C), followed by Az-fiber (green, ~290 °C), in line with previous
measurements. \textsuperscript{5b} Por\(^{(+)}\)-paper (red) also exhibited excellent thermostability, with substantial mass reduction only observed above 290 °C. Note that cellulose nanocrystals modified with Por\(^{(+)}\) (1), CNC-Por, \textsuperscript{5b} afforded a material with a similar thermal gravimetric behavior: minor degradation commencing around 210 °C (weight loss of <20%), with major decomposition above 320 °C. The Bdy\(^{(2I)}\)-paper was found to be the least thermostable, with significant weight loss occurring at ~250 °C.

\textbf{Figure 4.3.} Thermal gravimetric analysis of mother fibers (black), Az-fiber (green), Por\(^{(+)}\)-paper (red), and Bdy\(^{(2I)}\)-paper (blue).

\textbf{Antimicrobial Activity Results}

\textit{PDI Studies Employing Por\(^{(+)}\)-paper} – \textit{In vitro} aPDI studies employing the cationic porphyrin-based Por\(^{(+)}\)-paper were performed under fixed illumination conditions (30 min, 400-700 nm, 65±5 mW/cm\(^2\)) to enable comparisons with previous studies. \textsuperscript{5b, 5c} The two Gram-positive bacteria, \textit{S. aureus} ATCC-2913 and the vancomycin-resistant \textit{E. faecium} (VRE) strain ATCC-230, were found to be highly susceptible to photodynamic inactivation with Por\(^{(+)}\)-paper. \textit{S. aureus} was inactivated greater than 99.997+% (~5 log units; \(P<0.05\)) of the absorbed bacteria (Figure 4.4A). In contrast, under the same illumination conditions the bacteria exhibited 100% survival on the unmodified cellulose paper (PS-free control), demonstrating
the requirement of the photosensitizer for bacteria inactivation. Similar results employing the identical cationic photosensitizer conjugated to cellulose nanocrystals (Por(+)-CNCs) demonstrated a comparable level of inactivation (~6 log units) under identical conditions, and suggests that cellulose fibers are as effective a scaffold for photosensitizers as cellulose nanocrystals despite their lower surface area.\textsuperscript{5b, 5c} Por(+) -paper also proved highly efficient at inactivating vancomycin-resistant \textit{E. faecium} (ATCC-230) by 99.987\% (~4 log units, \(P<0.005\), Figure 4.4A). While this \textit{E. faecium} strain was not previously tested against Por(+) -CNCs, this bacterium has been shown to be highly susceptible to photodynamic inactivation (see Chapter 2).

Gram-negative bacteria are typically more resistant to photodynamic inactivation than Gram-positive species due to their additional outer membrane of highly impermeable lipopolysaccharides. Surprisingly, however, Por(+) -paper showed virtually identical efficacy against Gram-negative bacteria (Figure 4.4B) when compared to the Gram-positive strains above. Specifically, \textit{Acinetobacter baumannii} ATCC-19606 was photoinactivated by Por(+) -paper to 99.997\% reduction in CFU/mL (~5 log units; \(P<0.05\), \textit{Pseudomonas aeruginosa} ATCC-9027 to 99.97\% reduction (~4 log units; \(P<0.001\)), and the multi-drug resistant NDM-1-producing \textit{K. pneumoniae} clinical isolate ATCC-2146 strain explored here was inactivated to 99.994\% reduction (4.5 log units; \(P<0.05\)). Interestingly, while Por(+) -CNCs achieved a similar level of photoinactivation against \textit{A. baumannii}, they were less effective (<2.5 log units inactivation) against \textit{P. aeruginosa}, highlighting a difference between these two materials in favor of Por(+) -paper. The effects of extended photobleaching were examined by pre-illuminating the Por(+) -paper sheet 12 h, with the same illumination conditions, prior to running aPDI trials with \textit{A. baumannii} and no photobleaching or drop of in antimicrobial activity was noted, suggesting the material has excellent longevity. Overall, our results against both Gram-positive and Gram-negative bacteria are particularly noteworthy in the context of ‘ESKAPE’ (acronym for the most common nosocomial infectious agents, \textit{Enterococci faecium}, \textit{Staphylococcus aureus}, \textit{Klebsiella pneumoniae}, \textit{Acinetobacter baumannii}, \textit{Pseudomonas aeruginosa}, and \textit{Enterobacter} species) pathogens given the broad
photoinactivation efficacy of Por\textsuperscript{(+)}-paper against the bacterial strains examined here regardless of their drug-resistance phenotype.

**Figure 4.4.** Photodynamic inactivation studies employing Por\textsuperscript{(+)}-paper. (A) Gram-positive species: methicillin-susceptible \textit{S. aureus} (MSSA) ATCC-2913 and the vancomycin-resistant \textit{E. faecium} (VRE) ATCC-2320 strain. (B) Gram-negative species: \textit{A. baumannii} ATCC-19606, \textit{P. aeruginosa} ATCC-9027, and \textit{K. pneumoniae} ATCC-2146. The black bars represent the % survival of the illuminated PS-free control as a percent of the dark PS-free control, whereas the red bars represent the illuminated Por\textsuperscript{(+)}-paper as a percent of the dark control of Por\textsuperscript{(+)}-paper. For all bacteria, the illumination conditions were as follows: 30 min, 400-700 nm, 65±5 mW/cm\textsuperscript{2} (total fluence of 118 J/cm\textsuperscript{2}). As the plating technique employed to determine % survival did not allow for detection of survival rates of <0.0001\%, data points below the detection limit were set to 0.0001\% survival for graphing purposes. In the cases where error bars cannot be visualized, the error bars themselves were smaller than the marker employed in the plot.
**PDI Studies Employing Bdy\(^{(2H)}\)-paper and Bdy\(^{(2I)}\)-paper** – The boron-dipyrromethene-based Bdy\(^{(2H)}\)-paper and Bdy\(^{(2I)}\)-paper were also investigated for their aPDI efficacy. For Bdy\(^{(2H)}\)-paper, little to no significant inactivation of Gram-positive (Figure 4.5A) or Gram-negative (Figure 4.5B) bacteria was observed. This result was not unexpected as the lack of heavy atoms on the BODIPY photosensitizer scaffold leads to a high fluorescence quantum yield that is an alternative pathway (non-productive) to that of singlet oxygen generation. 15 Notably, this result serves to confirm that there is little to no non-photodynamic antimicrobial activity associated with these materials. As expected, the Bdy\(^{(2I)}\)-paper results were generally better than Bdy\(^{(2H)}\)-paper with the one notable exception that the vancomycin-resistant E. faecium (VRE) ATCC-2320 strain was not statistically different from the dark control. S. aureus ATCC-2913 was inactivated >90% (~1 log unit; \(P<0.01\)). Interestingly, Bdy\(^{(2I)}\)-paper proved more effective against the Gram-negative bacteria when compared to the Gram-positive ones: both A. baumannii ATCC-19606 and P. aeruginosa ATCC-9027 were inactivated with similar efficiency to 99.5% reduction in CFU/mL (2.5 log units). Though with limited statistical significance due to only two trials for the latter strain. K. pneumoniae ATCC-2146 was inactivated slightly less than the other two strains to ~90% reduction in CFU/mL (1 log unit; \(P<0.01\)). This may indicate a greater sensitivity of Gram-negative species than their Gram-positive counterparts to the neutral BODIPY-based photosensitizer, but this was not further explored as the overall efficacy of Bdy\(^{(2H)}\)-paper and Bdy\(^{(2I)}\)-paper was substantially poorer than Por\(^{(+)}\)-paper for all bacteria examined here.
Figure 4.5. Photodynamic inactivation studies employing Bdy\textsuperscript{(2H)}-paper and Bdy\textsuperscript{(2I)}-paper. (A) Gram-positive species: methicillin-susceptible \textit{S. aureus} (MSSA) ATCC-2913 and the vancomycin-resistant \textit{E. faecium} (VRE) ATCC-2320 strain. (B) Gram-negative species: \textit{A. baumannii} ATCC-19606, \textit{P. aeruginosa} ATCC-9027, and \textit{K. pneumoniae} ATCC-2146. The black bars represent the % survival of the illuminated PS-free control as a percent of the dark PS-free control, the dark blue bars represent the illuminated Bdy\textsuperscript{(2H)}-paper as a percent of the dark control of Bdy\textsuperscript{(2H)}-paper, and the light blue bars represent the illuminated Bdy\textsuperscript{(2I)}-paper as a percent of the dark control of Bdy\textsuperscript{(2I)}-paper. For all bacteria, the illumination conditions were as follows: 30 min, 400-700 nm, 65±5 mW/cm\textsuperscript{2} (total fluence of 118 J/cm\textsuperscript{2}). As the plating technique employed to determine % survival did not allow for detection of survival rates of <0.0001%, data points below the detection limit were set to 0.0001% survival for graphing purposes. In the cases where error bars cannot be visualized, the error bars themselves were smaller than the marker employed in the plot.

\textit{PDI Studies Employing Por\textsuperscript{(-)}-paper and Por\textsuperscript{(0)}-paper} – \textit{In vitro} aPDI studies were performed to examine the effects of charge on paper efficacy employing the anionic porphyrin-based Por\textsuperscript{(-)}-paper and the neutral porphyrin-based Por\textsuperscript{(0)}-paper. The materials were tested against both \textit{S. aureus} ATCC-2913 and \textit{A. baumannii} ATCC-19606 (Figure 4.6), but neither
material exhibited any noteworthy photodynamic inactivation efficacy against these strains, and additional studies were not pursued due to these poor aPDI results. Given that similar porphyrin-based photosensitizers possess reasonable photophysical properties, we surmise that the charge of the photosensitizer plays an important role in mediating the efficiency of PDI against bacteria in these PS-cellulose conjugates.
Figure 4.6. Photodynamic inactivation studies of methicillin-susceptible *S. aureus* (MSSA) ATCC-2913 and *A. baumannii* ATCC-19606 employing (A) Por($-1$)-paper and (B) Por($0$)-paper. The black bars represent the % survival of the illuminated PS-free control as a percent of the dark PS-free control, the green bars represent the illuminated Por($-1$)-paper as a percent of the dark control of Por($-1$)-paper, and the orange bars represent the illuminated Por($0$)-paper as a percent of the dark control of Por($0$)-paper. For both bacteria, the illumination conditions were as follows: 30 min, 400-700 nm, 65±5 mW/cm$^2$ (total fluence of 118 J/cm$^2$). As the plating technique employed to determine % survival did not allow for detection of survival rates of <0.0001%, data points below the detection limit were set to 0.0001% survival for graphing purposes. In the cases where error bars cannot be visualized, the error bars themselves were smaller than the marker employed in the plot.
Antiviral photodynamic inactivation studies employing Por\(^{(+)}\)-paper against dengue-1, influenza A, and human adenovirus-5 (HAd-5). The black bars represent the % activity of the illuminated paper-free control as a percent of the dark paper-free control, the green bars represent the % activity of the illuminated PS-free control as a percent of the dark PS-free control, and the purple bars represent the illuminated Por\(^{(+)}\)-paper as a percent of the dark control of Por\(^{(+)}\)-paper. The illumination conditions were as follows: 30 min, 400-700 nm, 65±5 mW/cm\(^2\) (total fluence of 118 J/cm\(^2\)). The shaded bars represent the detection limit (see Materials and Methods). In the cases where error bars cannot be visualized, the error bars themselves were smaller than the marker employed in the plot.

**Antiviral PDI Studies Employing Por\(^{(+)}\)-paper** – Por\(^{(+)}\)-paper was examined for its antiviral PDI activity against three viruses: dengue-1, influenza A, and human adenovirus-5 (HAd-5). No inactivation of any virus was seen in the absence of Por\(^{(+)}\)-paper (paper-free control) or in the presence of Whatman filter paper (PS-free paper control). However, in the presence of Por\(^{(+)}\)-paper, the dengue-1 virus was detection limit inactivated to >99.995% reduction in FFU/mL (4.5 log units; \(P<0.05\)) upon illumination. When investigated against the influenza A virus, Por\(^{(+)}\)-paper was again able to achieve detection limit inactivation, although due to a difference in starting concentration, the detection limit was ~99.5% reduction in FFU/mL (2.5 log units; \(P<0.001\)). Human adenovirus-5 proved the most difficult of the three viruses to inactivate, achieving ~99% reduction in FFU/mL (2 log units; \(P<0.01\)). It should be noted however, that there was more error associated with the human adenovirus-5 trials, and that the detection limit for this virus (3.5 log units) was well within one standard deviation of the average photodynamic inactivation.
4.5 Discussion

Antimicrobial materials employing a photodynamic mechanism of action have garnered increased attention over the last 5-10 y. \(^\text{17}\) We have previously reported on the synthesis, characterization, and efficacy of antimicrobial photodynamic inactivation of cellulose nanocrystals with a covalently attached cationic photosensitizer.\(^\text{5b, 5c}\) These studies demonstrated that uptake of the photosensitizer, whether into the membrane or cytoplasm, was not necessary to effectively inactivate bacteria, and that photosensitizers conjugated to a solid support could have excellent antimicrobial efficacy against a diverse range of pathogens. More recently, several other studies of photosensitizers attached to solid supports, including both synthetic polymers \(^\text{6a, 7b, 7c, 18}\) and a variety of cellulose-based materials, \(^\text{5a, 6b, 6c, 7a, 19}\) have been reported. With the increased attention to aPDI functional materials, there is an increased need to better understand their pathogen scope, mechanism of action, and to increase the scientific foundation to better understand their potential to combat infectious disease.

In this work, we report the preparation of five photosensitizer-conjugated paper sheets using the Cu(I)-catalyzed Huisgen-Meldal-Sharpless 1,3-dipolar cycloaddition or ‘Click’ reaction to conjugate ethyne-bearing photosensitizers to azide modified cellulose fibers (Az-fibers). Demonstrating the robustness of this strategy for preparing antimicrobial photodynamic cellulose materials, a range of photosensitizers was employed, including cationic, anionic, and neutral porphyrins, as well as two neutral BODIPY based photosensitizers, with the preparation of the PS-paper following the same chemistry previously reported for the covalent addition of photosensitizers to cellulose nanocrystals.\(^\text{5b}\) Notably, only minor solvent modifications were needed to accommodate the broader range of photosensitizers and the substitution of cellulose fibers (1-4 mm in length) here versus the single photosensitizer and cellulose nanocrystals (100-400 nm) used in that previous study. As revealed by thermal gravimetric analysis, the synthetic modifications had a minimal effect, with the thermal stability of the PS-paper sheets exceeding 250 °C, well below the threshold needed for applications in healthcare settings or the food packing industries which are largely envisioned to be at room temperature.
FTIR spectroscopy was used to assess the loading efficiency of each photosensitizer substrate by measuring the decrease in the azide band of the precursor Az-fiber that is correlated to the formation of the triazole linker between the photosensitizer and the cellulose fibers. Based on the near complete absence of an azide band for Bdy\textsuperscript{(2H)}-paper and Por\textsuperscript{(−)}-paper, ~50% reduction in band area for Bdy\textsuperscript{(2I)}-paper, and a ~30% reduction for Por\textsuperscript{(+)}-paper, the FTIR spectroscopic results suggested a loading efficiency of Bdy\textsuperscript{(2H)} \approx Por\textsuperscript{(−)} > Bdy\textsuperscript{(2I)} > Por\textsuperscript{(+)}. The efficiency of porphyrin loading was also assessed by Zn ICP-OES. Each free base porphyrin was metallated with zinc prior to the Cu(I)-catalyzed cycloaddition reaction to prevent undesired copper-metalation, as copper porphyrins exhibit extremely poor photophysical properties with respect to singlet oxygen generation. ICP-OES analysis revealed a porphyrin loading efficiency of Por\textsuperscript{(−)} > Por\textsuperscript{(+) > Por\textsuperscript{(0)},} with almost exactly the same ratio of Por\textsuperscript{(−)} to Por\textsuperscript{(+) (3:1)} observed by FTIR spectroscopy, suggesting that both measurements are accurate for determining photosensitizer loading efficiency. As Por\textsuperscript{(−)} is conjugated to the Az-fibers in its neutral, triprotic form, it is interesting to note that the trend in loading efficiency appears to suggest that neutral (or low charge density) precursor photosensitizers allow for more efficient loading. The results suggest that the poorer Por\textsuperscript{(+) loading may be attributable to electrostatic repulsion between a partially-loaded Por\textsuperscript{(+)}-paper and unloaded Por\textsuperscript{(+) (1)}, thereby limiting the conjugation reaction from going to completion. At the same time, the poorer loading of Por\textsuperscript{(0)} was likely due to steric hindrance by the dodecyl alkyl chains inhibiting interaction with either the copper catalyst or with the Az-fibers. We had originally hypothesized that stericus would be the primary determinant of photosensitizer loading efficiency, particularly between the smaller BODIPY compounds and the larger porphyrin ones, but the efficient loading of Por\textsuperscript{(−)} and the poor loading of Por\textsuperscript{(0)} suggest a more complicated interplay between charge and size, with both factors needing to be considered in the future design of photodynamic materials.

After the confirmation of photosensitizer loading, the electronic absorption spectra of the PS-paper conjugates were characterized by UV-vis diffuse reflectance spectroscopy. When compared with their alkyne-bearing precursors, each of the neutral PS-paper conjugates
(including the triprotic Por\(^{(+)}\)-paper) showed minor absorbance shifts (<5 nm) that can be attributed to the difference between solution and solid-state spectroscopy. The UV-vis spectrum of Por\(^{(+)}\)-paper showed a larger 10 nm bathochromic shift in the Soret band that can be attributed to a greater solvation effect on the highly charged porphyrin. A similar bathochromic shift was previously observed for Por\(^{(+)}\)-CNC when compared to the precursor Por\(^{(+)}\) (1),\(^c\) again likely due to reduced solvation of the photosensitizer on the surface of this material. Taken together, the UV-vis spectroscopic data suggest no significant absorption differences between the solution-based photosensitizers and the PS-paper materials that would preclude the use of the latter in subsequent aPDI studies (discussed below).

The results of the antibacterial PDI assay employing Por\(^{(+)}\)-paper demonstrated this material to be highly effective at inactivating bacteria. Though it had the second lowest photosensitizer loading (~30%), under the illumination conditions employed Por\(^{(+)}\)-paper was able to eliminate ~99.99% (~4 log units) of each of the bacterial strains tested. The material was also found to exhibit no decrease in antimicrobial activity, even after 12 h illumination. This virtually identical (within error) efficacy against a range of bacteria was particularly interesting in light of our previous results with Por\(^{(+)}\)-CNCs that showed a significant difference in photoinactivation between the bacteria. In that previous work, greater inactivation efficiency was observed for S. aureus and A. baumannii (~5+ log units reduction), but much lower inactivation was observed for E. coli (<2 log units) and P. aeruginosa (<3 log units). As to why Por\(^{(+)}\)-CNCs showed a differential response in antibacterial PDI and Por\(^{(+)}\)-paper did not, we suggest that the nature of the cellulose materials (paper vs. nanocrystals) themselves is the primary determinant. Por\(^{(+)}\)-CNC exhibited a strong dependence on the incubation time of the material in the bacterial culture (prior to illumination) for effective aPDI to be achieved, whereas no such incubation time dependence was observed here Por\(^{(+)}\)-paper (data not shown). The incubation time dependence of Por\(^{(+)}\)-CNC was likely due to it being suspended in the bacterial culture, and thereby led to an initially poor interaction between the photosensitizer and the bacteria. As singlet oxygen diffusion is limited to <250 nm, the inactivation of bacteria by these materials is restricted solely to their surface. Thus, as the adhesion/attraction
properties of the bacteria to the material surface is likely strain-dependent, the resulting photodynamic inactivation would be as well, leading to the strain-dependent photoinactivation differences observed for \textbf{Por}^{(+)}\text{-CNC}. By contrast, the interaction of \textbf{Por}^{(+)}\text{-paper} with bacteria was immediate as the bacterial culture was absorbed directly by the material, resulting in no strain-dependent properties governing their photodynamic inactivation were observed. Thus, \textbf{Por}^{(+)}\text{-paper} was found to be a highly effective material for the inactivation of the bacteria studied here regardless of their taxonomic designation.

Two additional porphyrin based PS-papers were prepared, \textbf{Por}^{(-)}\text{-paper} and \textbf{Por}^{(0)}\text{-paper}, to explore the effects of anionic charge and increased photosensitizer hydrophobicity, respectively. Although \textbf{Por}^{(-)}\text{-paper} exhibited the highest photosensitizer loading of the porphyrin-paper materials, in preliminary testing it showed less than a 1 log unit reduction in CFU/mL for \textit{S. aureus}, and no statistically significant inactivation of \textit{A. baumannii}. This result was not entirely unexpected given that electrostatic repulsion has been attributed to the comparatively poor performance of anionic photosensitizers as bacteria possess negatively charged cellular envelopes. As for \textbf{Por}^{(0)}\text{-paper}, this material exhibited the lowest level of photosensitizer loading, and was initially so hydrophobic that the bacterial solutions beaded on its surface, preventing the interaction of the material with the bacteria. To overcome this, the material was doped with mother fibers by 50%, yet this only served to dilute the photosensitizer loading even further, and in preliminary testing, no inactivation of either \textit{S. aureus} or \textit{A. baumannii} was observed. While the technical limitations prevented a thorough examination of \textbf{Por}^{(0)} at a loading efficiency similar to that of the other photosensitizers, it may be concluded from this study that \textbf{Por}^{(0)}\text{-paper} would be simply too hydrophobic for this particular application.

Previous studies with water-soluble BODIPY photosensitizers found them to be highly effective in solution.\textsuperscript{20} Given this, we prepared photosensitizer-conjugated paper sheets employing the BODIPY scaffold to determine their antimicrobial efficacy. The results of the antibacterial PDI assay employing \textbf{Bdy}^{(2I)}\text{-paper} demonstrated a more modest antibacterial efficacy than \textbf{Por}^{(-)}\text{-paper}, with the best results achieved against \textit{A. baumannii} and \textit{P.}
aeruginosa (~2.5 log units reduction in CFU/mL), slight inactivation of K. pneumoniae and S. aureus (~1 log unit reduction), and no inactivation of E. faecium. While the Bdy(2I) (#) photosensitizer employed here has a smaller singlet oxygen quantum yield than Por(+)(1), a lower yield of singlet oxygen production does not explain the lower efficacy of Bdy(2I)-paper when compared to Por(+)-paper given the higher photosensitizer loading efficiency of the former. As such, we suggest that the simplest explanation for the reduced efficacy of Bdy(2I)-paper was that the neutral and hydrophobic photosensitizer lacks the electrostatic attraction inherent in the cationic Por(+)-paper that appears to be needed for efficient inactivation. Interestingly, Bdy(2H)-paper exhibited greater efficacy against Gram-negative strains than Gram-positive ones that may be the result of an alternative secondary interaction between the material and the bacteria that is normally unobservable due to the large primary electrostatic attraction that dominates with cationic photosensitizers, however this was not further pursued given the overall low inactivation observed. Finally, Bdy(2H) was studied as a low singlet oxygen control: though structurally almost identical to Bdy(2I), the lack of the heavy atom effect in Bdy(2H) leads to it exhibiting a large fluorescence quantum yield, which is an alternative relaxation pathway to the excitation of singlet oxygen. As such, Bdy(2H)-paper showed little to no aPDI efficacy, likely due to a lack of singlet oxygen production.

Given the greater efficacy of Por(+)-paper against bacteria when compared to the other porphyrin- or BODIPY-based cellulose conjugates, we extended the aPDI application of this material in preliminary investigations against three viruses: dengue-1, influenza A, and human adenovirus-5. The results of the antiviral PDI assay employing Por(+)-paper demonstrated detection limit inactivation of both dengue-1 virus (4.5 log units) and influenza A (2.5 log units). Human adenovirus-5 was found to be slightly more resistant to photoinactivation, but a notable 99% reduction in FFU/mL (2 log units) was still achieved. As human adenovirus-5 was the only non-enveloped virus examined, this result may suggest that the protein-based capsids of these viruses are more resistant to photosensitization than lipid-bilayer enclosed viruses. While some mechanistic studies of antiviral PDI have been reported, further studies of non-enveloped viruses would be necessary to firmly establish this. Moreover, additional
studies employing neutral and anionic photosensitizers will be needed to better understand the scope of antiviral PDI with these materials, as electrostatic effects are anticipated to be less of a factor for viruses than for bacteria. Overall, however, our preliminary study here demonstrates that materials such as $\text{Por}^{(+)}$-paper show promise for their application in antiviral PDI.

4.6 Conclusions

Using the robust Huisgen-Meldal-Sharpless 1,3-dipolar cycloaddition reaction, five diverse photosensitizers were covalently attached to cellulose fibers and subsequently pressed into PS-paper sheets. FTIR and ICP-OES Zn analysis demonstrated the successful addition of each photosensitizer, which was in some cases discovered to be nearly quantitative. While it was found that the size difference between porphyrins and BODIPY based photosensitizers did not affect loading efficiency, the addition of bulky alkyl chains to $\text{Por}^{(0)}$ (9) appeared to sterically hinder the reaction, leading to lower loading efficiency. The results also suggested that high photosensitizer charge density may reduce loading efficiency, though to a much lesser degree. After thorough characterization with UV-vis DRS and TGA, the antimicrobial efficacies of the sheets were determined using a newly designed aPDI assay for surface materials. This assay was thoroughly controlled to elucidate reductions in microbial concentrations caused exclusively by aPDI. It was used to test the prepared PS-papers against taxonomically diverse bacteria including: $S$. aureus, $E$. faecium, $A$. baumannii, $P$. aeruginosa, and $K$. Pneumoniae. The prepared cationic porphyrin modified paper, $\text{Por}^{(+)}$-paper, was found to have broad antibacterial efficacy, resulting in $\sim 4$ log units inactivation of each of the bacterial strains tested. Investigations of neutral and anionic PS-papers, including both porphyrin and BODIPY photosensitizers, showed greatly reduced efficacies in comparison, suggesting that cationic charges are crucial for antibacterial efficacy. Since $\text{Por}^{(+)}$-paper was found to be the most effective material against bacteria, its antimicrobial studies were extended to viruses. It was again found to be highly effective, causing detection limit inactivation of both dengue-1 virus and influenza A virus, while inactivating 2 log units of human adenovirus-5. The combined
antimicrobial results suggest a great potential for \textbf{Por}^{(+)}-paper as well as future iterations of these materials to prevent the transmission of infectious disease through the sterilization of surfaces, whether they be used in the food packaging industry, hospital environments, or used for other sterilization applications. The conjugation strategy employed was found to be highly effective on a diverse group of photosensitizers, as well as cellulose materials of widely varied scales. Our study suggests that future iterations of these materials should be designed with cationic charges to be effective against bacteria, though it was not determined whether these charges must be exclusively on the photosensitizer scaffold or how they affect antiviral activity. Also, since the neutral BODIPY based \textbf{Bdy}^{(2H)}-paper outperformed the neutral porphyrin paper Por^{(0)} (9), future synthesis and addition of a cationic BODIPY based photosensitizer may prove highly efficacious.

4.7 References


