

Polymeric systems for antimicrobial photodynamic therapy of bacterial wound infections

DISSERTATION

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This Thesis is dedicated to my family, who have always supported me in my adventures.

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Abbreviations and acronyms

$\varnothing$	diameter	Mn	number-average molecular weight
A	absorbance	Mw	weight-average molecular weight
a.u.	arbitrary units	mPEG	PEG methyl ether
a_0	effective interfacial area	MRD	maximum recovery diluent
ABCP	amphiphilic block copolymers	MRSA	methicillin-resistant <i>Staphylococcus aureus</i>
AHL	<i>N</i> -acyl homoserine lactone	N	degree of polymerisation
AMR	antimicrobial resistance	n	refractive index
ANOVA	analysis of variance	NMR	nuclear magnetic resonance
aPDT	antimicrobial photodynamic therapy	p	packing parameter
ATR-FTIR	attenuated total internal reflection Fourier transform infrared	<i>P. aeruginosa</i>	<i>Pseudomonas aeruginosa</i>
ATRP	atom transfer radical polymerisation	PAA	poly(acrylic acid)
b	block	PBS	phosphate buffer solution
CFU	colony-forming unit	PCL	poly(ϵ -caprolactone)
cru	constitutional repeating units	PDT	photodynamic therapy
CS	chitosan	PEG	poly(ethylene glycol)
D	diffusion coefficient	PEO	poly(ethylene oxide)
D	dispersity	PET	photoinduced electron transfer
DLS	dynamic light scattering	PGA	poly(glycolic acid)
<i>E. coli</i>	<i>Escherichia coli</i>	PhB	Phloxine B
EPS	extracellular polymeric substance	PHEMA	poly(2-hydroxyethyl methacrylate)
FBS	foetal bovine serum	Pheo	pheophorbide α
FDA	U.S. Food and Drug Administration	PLA	poly(lactic acid)
FESEM	field emission scanning electron microscopy	PS	photosensitiser
GPC	gel permeation chromatography	PTT	photothermal therapy
HpD	haematoporphyrin derivative	PU	polyurethane
I	fluorescence intensity	QD	quantum dot
ISC	intersystem crossing	R6G	Rhodamine 6G
J	coupling constant (Hz) in ^1H NMR spectra	RAFT	reversible addition fragmentation chain transfer
JIS	Japanese Industrial Standards	RI	refractive index
l	length	ROP	ring-opening polymerisation
L	liquid	ROS	reactive oxygen species
LB	lysogeny broth	rt	room temperature
LED	light-emitting diode	S	solid
LOD	limit of detection	<i>S. aureus</i>	<i>Staphylococcus aureus</i>
LRF	log reduction factor	SANS	small-angle neutron scattering

SAXS	small-angle X-ray scattering	V	vapour
SDS	sodium dodecyl sulfate	Wd	dry mass
S_n	singlet state	Ws	swollen mass
Sn(Oct)₂	tin(II) octoate	γ	interfacial surface tension
SOSG	Singlet Oxygen Sensor Green	Γ	surface concentration
STWE	simulated thin wound exudate	δ	chemical shift (ppm) in NMR spectra relative to TMS
SWCA	static water contact angle	ε	extinction coefficient
SWF	simulated wound fluid	θ	contact angle
t_{1/2}	half life	λ_{em}	emission wavelength
TEM	transmission electron microscopy	λ_{ex}	excitation wavelength
Tg	glass transition temperature	φ_F	fluorescence quantum yield
T_n	triplet state	φ_Δ	¹ O ₂ quantum yield
UV-Vis	ultraviolet-visible	χ	Flory-Huggins interaction parameter
v	volume		

Abstract

Antimicrobial photodynamic therapy (aPDT) is a promising alternative to conventional antibiotics for treating and preventing microbial infections, owing to its broad-spectrum activity and low potential for resistance development. For application in future advanced wound dressings, photosensitisers (PSs) must be immobilised or encapsulated to maintain a high local concentration at the site of infection. This Thesis investigates the incorporation of the FDA-approved xanthene dye Phloxine B (PhB) as a PS for aPDT into polymer-based delivery systems. The first part of this work examines the fundamental photophysical behaviour of PhB, including its absorption and emission, pH-dependence, $^1\text{O}_2$ generation and photostability. The aPDT activity of PhB was systematically evaluated against Gram-positive *Staphylococcus aureus* (*S. aureus*) and Gram-negative *Pseudomonas aeruginosa* (*P. aeruginosa*), common pathogens found in wound infections, establishing its efficacy as an aPDT agent. Building on these findings, the second part of this Thesis focuses on the synthesis and characterisation of amphiphilic poly(ethylene glycol)-*block*-polyester (PEG-*b*-polyester) copolymers and their self-assembly into nanoscale assemblies for PhB encapsulation. Copolymers with varying hydrophobic block lengths formed assemblies with diameters ranging from 50-200 nm. As a water-soluble PS, PhB was encapsulated into polymeric vesicles ~200 nm in diameter, achieving dye concentrations >30 μM in the vesicle suspensions. Encapsulated PhB retained its aPDT activity, successfully eradicating both *S. aureus* and *P. aeruginosa* after 15 min of green light irradiation. The timescales of the antibacterial studies suggested at most limited internalisation of PhB, motivating the development of immobilised PhB-chitosan (CS) films. The final part of the Thesis therefore explores these PhB-CS films as aPDT surfaces, demonstrating effective inactivation of both planktonic and biofilm-associated bacteria upon light activation. Overall, this Thesis advances the integration of PhB into functional polymeric materials for application in advanced wound dressings.

Zusammenfassung

Für die Behandlung und Prävention mikrobieller Infektionen stellt die antimikrobielle photodynamische Therapie (aPDT) aufgrund des breiten Wirkspektrums und des geringen Potenzials zur Resistenzentwicklung eine vielversprechende Alternative zu herkömmlichen Antibiotika dar. Für die Anwendung in zukünftigen fortschrittlichen Wundauflagen müssen Photosensibilisatoren (PS) immobilisiert oder eingekapselt werden, um eine hohe lokale Konzentration an der Infektionsstelle aufrechtzuerhalten. In dieser Arbeit wurde die Anwendbarkeit des von der FDA zugelassenen Xanthenfarbstoffs Phloxine B (PhB) als PS für die aPDT in polymerbasierten Trägersystemen untersucht. Im ersten Teil dieser Arbeit wurde das grundlegende photophysikalische Verhalten von PhB, einschließlich des Absorptions- und Emissionsverhalten, die pH-Abhängigkeit, die Erzeugung von $^1\text{O}_2$ sowie die Photostabilität erforscht. Die aPDT-Aktivität von PhB wurde systematisch gegen grampositive *Staphylococcus aureus* (*S. aureus*) Stämme sowie einen gramnegativen *Pseudomonas aeruginosa* (*P. aeruginosa*) Stamm, häufige Erreger von Wundinfektionen, bewertet, um die Wirksamkeit als aPDT-Wirkstoff nachzuweisen. Aufbauend auf diesen Ergebnissen wurden im zweiten Teil dieser Arbeit amphiphile Poly(ethylenglykol)-*block*-Polyester (PEG-*b*-Polyester)-Copolymere synthetisiert, charakterisiert und deren Selbstorganisation zu nanoskaligen Aggregaten zur Einkapselung von PhB bewertet. Copolymere mit unterschiedlichen hydrophoben Blocklängen wurden verwendet, um Aggregate mit Durchmessern zwischen 50 und 200 nm zu bilden.

Als wasserlöslicher PS wurde PhB in polymere Vesikel mit einem Durchmesser von ~200 nm eingekapselt, wodurch Farbstoffkonzentrationen von >30 μM in den Vesikelsuspensionen erreicht wurden. Das eingekapselte PhB behielt seine aPDT-Aktivität bei und tötete sowohl *S. aureus* als auch *P. aeruginosa* nach 15-minütiger Bestrahlung mit grünem Licht erfolgreich ab. Die Zeitskalen der antibakteriellen

Studien deuteten auf eine limitierte Internalisierung von PhB hin, was zur Entwicklung von immobilisierten PhB-Chitosan (CS)-Filmen motivierte. Im letzten Teil dieser Arbeit wurden die zuvor beschriebenen PhB-CS-Filme als aPDT-Oberflächen untersucht, und es wurde gezeigt, dass sowohl planktonische als auch Biofilm-assoziierte Bakterien nach Lichtaktivierung des PS inaktiviert werden konnten. Insgesamt demonstriert diese Arbeit die Integrierbarkeit von PhB in funktionelle Polymermaterialien für die Anwendung in modernen Wundauflagen.

1. Introduction & motivation

Wound healing is a highly regulated and dynamic biological process that proceeds through four phases: haemostasis, inflammation, proliferation and remodelling.¹ Disruption of this sequence, whether due to impaired perfusion, metabolic disease, repeated trauma, or microbial burden, can prevent timely resolution and lead to the formation of a chronic wound.² Chronic wounds are clinically defined as wounds that fail to progress through the normal stages of healing within an expected timeframe and represent a major and growing healthcare challenge worldwide.³

A defining feature of all wounds is the presence of microorganisms. Immediately following tissue damage, bacteria originating from the patient's microbiota or the surrounding environment contaminate the wound surface.⁴ In many cases, this microbial presence remains controlled by host immune responses and does not impede healing or cause infection.⁴ However, as bacterial load increases beyond a threshold level, commonly cited as 10^5 colony-forming units (CFUs) per gram of tissue, host defences are overwhelmed and wound healing is impaired.⁴ An infection develops when a sufficiently large bacterial population colonises the wound bed and begins to proliferate.⁵

While acute infections can often be managed with antibiotics, chronic infections pose a significantly greater treatment challenge.^{3,6,7} In the United States alone, approximately 2.5% of the population currently suffers from a chronic wound, with incidence expected to increase due to ageing populations and increasing rates of diabetes and vascular disease.⁸ Importantly, a significant proportion of these wounds become infected, with infection rates in these wounds reported to be around 22%.^{8,9} These infections are associated with delayed healing, increased rates of hospitalisation and amputation and markedly increased healthcare costs.¹⁰ The economic burden of chronic wound management is tens of billions of dollars

annually in the United States and Europe, highlighting an urgent need for improved therapeutic strategies.¹¹

Management of wound infections relies primarily on antimicrobial agents, including antiseptics and antibiotics.^{4,12} Antiseptics such as alcohols, biguanides and silver-based compounds exhibit broad-spectrum activity but act through non-specific cytotoxic mechanisms and are primarily used to prevent infection rather than for long-term treatment.⁴ In contrast, antibiotics target specific bacterial pathways and are widely used in both topical and systemic formulations.¹³ Systemic antibiotic administration remains common in wound care despite the risk of adverse side effects; one study reported that over 60% of patients with chronic wounds received at least one course of antibiotics.⁴

The widespread and often prolonged use of antibiotics has contributed to the emergence of antibiotic-resistant bacteria, which now represent a critical threat to effective wound management.¹⁴ Resistant strains such as methicillin-resistant *Staphylococcus aureus* (*S. aureus*) (MRSA) and multidrug-resistant *Pseudomonas aeruginosa* (*P. aeruginosa*) are frequently isolated from chronic wounds and are associated with poor clinical outcomes.¹⁵ The first reported case of vancomycin-resistant *S. aureus* (VRSA), isolated from a chronic wound patient in the United States over 20 years ago, exemplifies the ability of wound environments to facilitate resistance development.¹⁶ While antibiotic resistance is a complex phenomenon, its clinical consequences are clear. Treatment options are increasingly limited, more toxic second-line drugs are required, and the risk of treatment failure continues to rise.¹⁷ This has prompted urgent calls by the World Health Organisation for antimicrobial strategies that are effective yet do not rely on conventional antibiotic mechanisms.¹⁸

In response to these challenges, significant research efforts have focused on identifying alternative antimicrobial therapies, including antimicrobial peptides, bacteriophages, probiotics, antibody-antibiotic conjugates and

immunotherapies.^{19–22} Among these approaches, antimicrobial photodynamic therapy (aPDT) has emerged as a particularly promising alternative.^{23–25} aPDT employs a photosensitiser (PS), which, upon activation by light in the presence of molecular oxygen, generates reactive oxygen species (ROS).²⁶ These ROS induce oxidative stress in bacterial cells, resulting in cell death.²⁵

Unlike antibiotics, which typically target a specific bacterial mechanism, aPDT inflicts non-specific oxidative damage, disrupting multiple metabolic pathways, reducing the potential for resistance development.²⁵ To date, repeated aPDT treatments have not been reported to select for resistant strains.²⁷ Importantly, as ROS are short-lived and generated only upon light activation, aPDT can be spatially and temporally controlled.²⁸ Minimising direct cytotoxicity toward host cells is particularly critical in the wound environment, where excessive damage to keratinocytes, fibroblasts or endothelial cells would impair healing.²⁹

PSs used in aPDT are commonly classified into three generations. First-generation PSs were limited by poor water solubility, poor light absorption and undesirable toxicity.³⁰ In the 1990s, second-generation PSs addressed many of these limitations, with improved stability and enhanced photosensitivity, the most significant advance being the ability to absorb longer-wavelength light, enabling deeper tissue penetration.³¹ More recently, third-generation systems have focused on improving bioavailability and localisation by combining second-generation PSs with targeting moieties or delivery vehicles.³²

Historically, PDT has been most extensively investigated in oncology, where delivery strategies were designed to promote PS accumulation in tumour tissue.³³ Passive carriers such as liposomes, hydrophilic polymer-PS conjugates, and biodegradable polymeric particles exploit the natural passive diffusion and phagocytosis processes.³³ One example, the liposomal formulation CGP55847, a Zinc (II) phthalocyanine-loaded carrier composed of POPC and DOPS developed by Novartis, entered phase I/II clinical trials for the treatment of squamous cell

carcinoma in the 1990s but was discontinued in 2003.³⁴ Polymeric carriers offer several advantages over lipid-based systems, including higher loading capacity, improved stability and the potential for tailoring materials to control degradation rate and surface functionality.³⁵ Amphiphilic block copolymers, first proposed as drug-delivery systems by Ringsdorf in 1984, can self-assemble into nanostructures such as vesicles and micelles by exploiting differences in solubility between the two blocks in selective solvents, providing versatile platforms for drug encapsulation.^{36,37} Notably, Pluronic, block copolymers of poly(ethylene oxide) and poly(propylene oxide), have been extensively studied as low-toxicity drug delivery vehicles.^{36,38} PEGylated vesicles, in particular, are well-reported to improve vesicle stability and biocompatibility.³⁹

For wound care applications, biodegradable polymers are especially attractive as they contain labile covalent bonds which can be degraded into non-toxic byproducts.⁴⁰ Polyesters such as poly(lactic acid), poly(ϵ -caprolactone) and poly(3-hydroxybutyrate) degrade primarily through hydrolytic cleavage of ester bonds and are widely used in biomedical applications.^{38,41} Beyond passive delivery, such biodegradable systems enable the development of stimuli-responsive platforms in which environmental cues, such as changes in pH, temperature, or pathogen-derived chemical signals, can trigger polymer degradation and release of encapsulated drugs.^{38,42}

In parallel with nanoparticle-based delivery systems, immobilisation of photosensitisers on solid supports represents an alternative strategy for local antimicrobial therapy.⁴³ By conjugating a PS to wound dressing materials, contact-active antimicrobial surfaces can be generated, reducing the risk of systemic exposure.^{5,44} Biopolymers such as chitosan are of particular interest as support materials for wound dressings due to their natural origin, biocompatibility and chemical versatility.⁴⁵

2. State of knowledge

2.1. Antimicrobial resistance in infected wounds

2.1.1. Burden of chronic and infected wounds

Wounds arise from disruption of the skin barrier and can be broadly classified as acute or chronic. Acute wounds typically result from trauma or surgery and proceed through an orderly healing process, whereas chronic wounds often arise from underlying pathological conditions such as diabetes, vascular disease or prolonged pressure.^{46,47} Wound healing is a complex, multistage cascade with four distinct phases: haemostasis, inflammation, proliferation and tissue remodelling, all of which are required to restore tissue integrity.⁴⁶

The presence of high bacterial loads within a wound can interfere with this healing process, leading to prolonged inflammation, delayed closure, increased patient morbidity and, in severe cases, mortality.^{5,46} During the inflammatory stage, neutrophils infiltrate the wound site and contribute to bacterial clearance through phagocytosis, degranulation and the generation of ROS.⁴⁶ While these mechanisms are essential for host defence, excessive or prolonged inflammation can damage host tissue and further impair healing, particularly in chronic wounds.⁴⁶

Multiple bacterial species can colonise chronic wounds at any one time. In an 8-week study of chronic venous leg ulcers, 2-5 bacterial species were detected in 76% of wounds.⁴ The most prevalent pathogens associated with infected wounds are *Staphylococcus aureus* (*S. aureus*) and *Pseudomonas aeruginosa* (*P. aeruginosa*). A clinical survey conducted in an Italian hospital reported that 37% and 17% of infected wounds contained *S. aureus* and *P. aeruginosa*, respectively.⁴⁸ Polymicrobial infections are common, with *S. aureus*/*P. aeruginosa* co-infections occurring frequently and often associated with delayed healing and increased treatment failure.^{48,49} These aerobic bacteria in chronic wounds promote local

tissue hypoxia through oxygen consumption, creating conditions that favour colonisation by anaerobic microorganisms.⁴⁹ As a result, chronic wounds often harbour higher numbers of anaerobes than acute wounds.⁴ Common anaerobic genera include *Prevotella*, *Bacteroides*, *Peptostreptococcus*, and *Porphyromonas*, all of which have been implicated in impaired healing and chronic inflammation.⁴

2.1.2. Clinically relevant wound pathogens

Staphylococcus aureus

S. aureus is a Gram-positive, aerobic coccus and a common constituent of the normal human skin and nasal microbiota, present in approximately 30% of the adult population.^{50,51} Individual cells are typically ~1 μm in diameter.⁵² Despite its commensal status, *S. aureus* is a leading cause of skin, soft tissue, bone, and blood infections, with most infections of endogenous origin.^{53,54} It is also a major cause of nosocomial infections, particularly in surgical wounds, from which it can disseminate systemically.^{51,52}

The pathogenicity of *S. aureus* is mediated by a diverse arsenal of virulence factors, including surface-associated adhesins that promote attachment to host tissues, secreted toxins that damage host cells, and capsular components that enable evasion of phagocytosis.⁵² Antibiotic resistance is widespread, with methicillin-resistant *S. aureus* (MRSA) representing a significant clinical challenge.⁵² MRSA strains express an altered penicillin-binding protein, conferring resistance to β -lactam antibiotics, including cephalosporins and carbapenems.⁵²

Several *S. aureus* toxins play a direct role in wound pathology. α -toxin, secreted by 85% of *S. aureus* clinical isolates, forms pores in host cell membranes, disrupting ionic homeostasis and leading to cell death.⁵⁴ In the context of wound infection, the extracellular fibrinogen-binding protein, Fib, has been linked to increased disease severity and delayed healing, as it promotes bacterial adhesion to fibrin-rich wound matrices.⁵⁴

Pseudomonas aeruginosa

P. aeruginosa is a Gram-negative, aerobic bacillus commonly found in moist environments.⁵² Cells typically measure 0.5-0.8 µm x 1.5-3.0 µm and are motile due to the presence of flagella.⁵² While infections are rare in healthy individuals, *P. aeruginosa* poses a significant threat to immunocompromised patients, including those with burns, cancer, cystic fibrosis, or chronic wounds.⁵² *P. aeruginosa* infections are notoriously difficult to treat due to their intrinsic and acquired antibiotic resistance mechanisms, metabolic versatility, and strong propensity for biofilm formation.⁵⁰

P. aeruginosa can utilise a wide range of organic sources and survives under hypoxic conditions by using alternative electron acceptors such as nitrate, which is particularly relevant in oxygen-depleted wound environments.^{25,52} *P. aeruginosa* is also able to tolerate high salt concentrations, grow at temperatures up to 42 °C, and frequently contaminates medical equipment, contributing to its role as a nosocomial pathogen.^{25,52} Approximately 90% of strains secrete proteases that degrade host tissues, further exacerbating wound damage.⁵²

2.1.3. Biofilms in wound care

A major challenge in the management of chronic and infected wounds is the persistence of biofilms: surface-associated microbial communities embedded within a self-produced extracellular polymeric substance (EPS) matrix.⁴ Biofilms adhere strongly to wound tissue and dressing materials, forming a dynamic, self-renewing barrier that protects bacteria from antimicrobial agents and host immune responses.^{4,55} Furthermore, these systems can mature and rupture, releasing planktonic bacteria into the environment, which cause secondary infections.⁵⁶

The EPS matrix contains polysaccharides, proteins, extracellular DNA and open water channels that facilitate nutrient transport and removal of metabolic waste products (Figure 1).^{4,57,58} Critically, this matrix acts as a physical and chemical

barrier, reducing antimicrobial penetration and promoting altered metabolic states associated with tolerance.⁵⁹ Bacteria within biofilms can exhibit up to 500× greater resistance to antibiotics than their planktonic counterparts.⁴ Slower growth rates and reduced metabolic activity of cells in biofilms further limit the efficacy of conventional antibiotics due to decreased drug uptake.^{4,60}

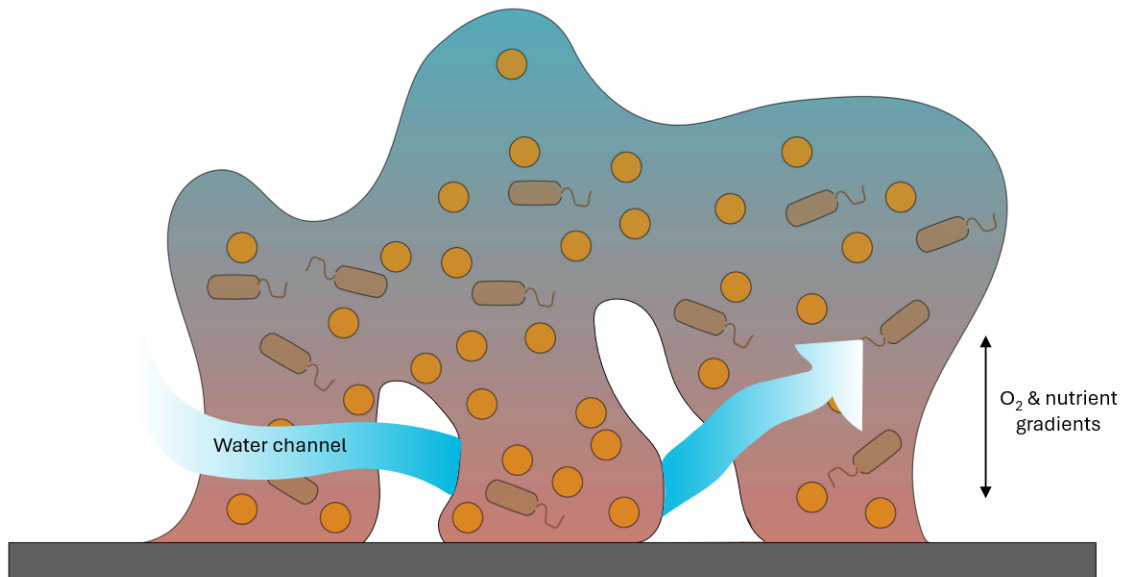


Figure 1. Biofilms are composed of bacteria surrounded by EPS, with metabolic gradients that lead to heterogeneity.

Biofilm formation is coordinated through quorum sensing, a cell-cell communication mechanism mediated by signalling molecules such as *N*-acyl homoserine lactones (AHLs).⁶¹ These systems regulate virulence, EPS production and collective behaviour.^{61,62} Chronic wound beds provide an ideal environment for biofilm establishment due to the presence of exposed extracellular matrix proteins, necrotic tissue, and persistent inflammation.⁴ Both *S. aureus* and *P. aeruginosa* are prolific biofilm producers, with *P. aeruginosa* particularly renowned for forming dense, highly resilient biofilms.⁴

From a materials perspective, biofilms formed at the solid–liquid interface severely limit the efficacy of passive antimicrobial dressings, motivating the development of active, non-traditional antimicrobial strategies.⁶³

2.1.4. Antimicrobial resistance and limitations of current strategies

The global rise of antimicrobial resistance (AMR) poses a critical threat to public health. Projections estimate that by 2050, AMR could result in up to 10 million deaths annually worldwide, surpassing mortality from cancer and road traffic accidents.⁶⁴ The burden is expected to be greatest in Asia and Africa, with estimated annual deaths of 4.7 million and 4.15 million, respectively.⁶⁴ Economically, AMR is projected to cost between USD 300 billion and 1 trillion per year due to increased healthcare expenditure and productivity losses.⁶⁴

Current wound care strategies rely heavily on systemic antibiotics and passive antimicrobial dressings. However, systemic antibiotics often fail to achieve effective concentrations within poorly perfused wound tissue and are particularly ineffective against biofilm-associated infections.⁶⁵ Passive antimicrobial dressings, such as those incorporating silver or iodine, provide a limited duration of activity and may contribute to cytotoxicity or resistance selection. Antibiotics act as a selection pressure, leading to the survival of resistant cells, which can either degrade the drug, alter the target of the drug, or prevent endocytosis of the drug (Figure 2).⁶⁶ These surviving cells then multiply and pass on their resistance genes, resulting in a community of resistant bacteria that can evade the effects of antibiotics.⁶⁷

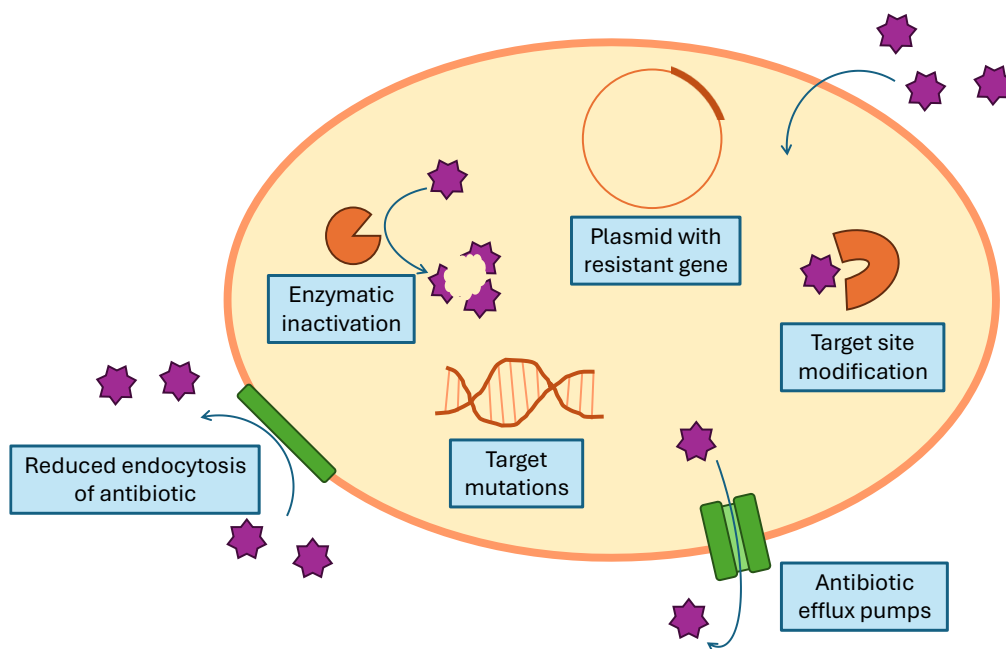


Figure 2. Mechanisms of antibiotic resistance within a bacterial cell.

Although bacteria evolved to tolerate antibiotics before their introduction into the environment, the rise in antibiotic use has provided unprecedented selection pressures.^{67,68} In one study, up to 50% of all *S. aureus* isolates from leg ulcers were methicillin-resistant (MRSA), while over a third of *P. aeruginosa* isolates exhibited resistance to ciprofloxacin.⁴ Treating severe *S. aureus* infections now relies on expensive second-line drugs, which often have severe side effects, necessitating monitoring during treatment and imposing a substantial economic burden.¹⁸

Recognising the growing threat, the World Health Organisation warned in 2014 of a post-antibiotic era in which even common infections could again become fatal.¹⁸ There is therefore an urgent need for alternative antimicrobial strategies that minimise the development of resistance, are effective against biofilms, and can be integrated into wound dressings.⁶⁹ Emerging approaches include antimicrobial peptides, bacteriophage therapy, host-directed therapies, and light-activated antimicrobial systems.⁷⁰ Among these, photodynamic antimicrobial strategies offer a promising route to localised, on-demand bacterial eradication without reliance on conventional antibiotics.

2.1.5. Design requirements for wound dressings

Traditional wound dressings primarily function as physical barriers that protect the wound from the external environment. In contrast, effective antimicrobial wound dressings are required to not only provide protection but also to actively promote healing while preventing or eradicating infection. To meet these demands, and depending on the wound type, a wide range of dressing architectures have been developed, including hydrogels, polymeric films, fibrous mats and porous sponges.⁴⁹ These materials provide versatile substrates for the incorporation of advanced antimicrobial strategies, but must meet the criteria to effectively manage the wound, including biocompatibility, moisture maintenance and encouraging gas exchange.^{71,72}

Biocompatibility is a critical requirement for any wound-contacting material. Dressings must not induce cytotoxicity, excessive inflammation or adverse immune responses, particularly given their prolonged contact with damaged tissue. Consequently, materials intended for use in wound dressings should be non-toxic, non-immunogenic, and compatible with the dynamic biological environment.⁷³

Maintaining a moist wound environment is widely recognised as essential for optimal healing, as it promotes epithelialisation and supports cellular migration.⁷⁴ Modern wound dressings are therefore designed to retain moisture while allowing gas exchange and managing wound exudate. Antimicrobial strategies integrated into such dressings must therefore remain effective under hydrated conditions and in the presence of wound fluid.

An increasingly important consideration is the development of non-leaching antimicrobial strategies.⁶³ While dressings incorporating diffusible antimicrobials can provide initial efficacy, uncontrolled release often leads to rapid depletion of the active component, reduced long-term effectiveness, and potential cytotoxicity to host tissue.⁶³ In contrast, surface-bound or immobilised antimicrobials offer the

potential for sustained, localised activity without uncontrolled release into the wound bed.

Accordingly, advanced wound care technologies increasingly seek to incorporate biocompatible antimicrobial coatings that actively modulate the wound environment by maintaining moisture, absorbing exudate, and delivering active ingredients to combat infection.⁴⁹ Systems that provide on-demand antimicrobial activity in a spatially and temporally controlled manner are particularly attractive for chronic wound management, where repeated interventions may be required without frequent, painful dressing changes. In this context, light-triggered antimicrobial approaches, such as aPDT, are particularly well-suited to wound infection applications as they provide both spatial and temporal control.

2.2. Light-triggered antimicrobial strategies

Light-triggered antimicrobial strategies have emerged as a powerful class of on-demand approaches for infection control, offering high spatial and temporal precision while minimising systemic exposure. By confining antimicrobial activity to the illuminated region, light-responsive systems enable localised treatment of infected tissue without continuous release of bioactive agents. This is particularly advantageous in wound care, where the infected area is readily accessible to external light sources and repeated activation can be achieved without disturbing the dressing.

While often neglected in reports on alternatives to antibiotics, leading scientists in the field have called for greater attention to light-based approaches.⁷⁵ Several distinct light-activated antimicrobial modalities have been developed, including photothermal therapy (PTT), photodynamic therapy (PDT), and photocleavable or photo-responsive release systems. Each exploits a different photophysical

mechanism to induce bacterial killing and presents unique advantages and limitations for wound applications.

2.2.1. Photothermal systems

Photothermal antimicrobial strategies rely on the conversion of absorbed light into heat, typically using plasmonic nanoparticles (e.g. gold nanostructures), carbon-based materials, or semiconducting polymers. Upon irradiation, localised temperature increases can disrupt bacterial membranes, denature proteins, and ultimately cause cell death.⁷⁶ Photothermal approaches offer rapid killing and are effective against both planktonic bacteria and biofilms; however, precise temperature control is required to avoid thermal damage to surrounding healthy tissue and sub-lethal heating, which can induce tolerance.⁷⁶ These limitations are particularly relevant in chronic wounds, where tissue is already compromised by prolonged inflammation and impaired vascularisation.⁷⁷

2.2.2. Photocleavable systems

An alternative approach involves photocleavable antimicrobial systems, in which light exposure triggers the cleavage of chemical bonds to release an antimicrobial agent from a carrier or surface.⁷⁸ These systems offer temporal control over drug release and can reduce premature depletion of the active compound.⁷⁹ Nevertheless, they still rely on diffusible antimicrobials and may suffer from limited penetration into dense biofilms, as well as challenges associated with incomplete cleavage or by-product formation.

2.2.3. Photodynamic systems

Among light-responsive strategies, antimicrobial photodynamic therapy (aPDT) has attracted considerable interest for wound applications.⁸⁰ In aPDT, a photosensitiser (PS) is activated by light of an appropriate wavelength in the presence of molecular oxygen, generating ROS, most notably singlet oxygen ($^1\text{O}_2$).⁸¹ These short-lived

species oxidise essential cellular components, including lipids, proteins, and nucleic acids, leading to rapid bacterial inactivation.

Among these light-triggered strategies, photodynamic therapy has emerged as a promising strategy for wound dressings. A key advantage of aPDT is its multitarget, non-specific mechanism of action, which makes the development of microbial resistance highly unlikely. Unlike conventional antibiotics, aPDT does not rely on a single molecular target (e.g. vancomycin binding to the D-Ala-D-Ala residues to inhibit peptidoglycan synthesis), and is effective against a broad spectrum of microorganisms, including Gram-positive and Gram-negative bacteria, antibiotic-resistant strains, and biofilm-associated cells.²⁵ Furthermore, ROS generation is confined to the vicinity of the PS and the illuminated region, providing inherent spatial control and limiting off-target effects.⁸²

From a wound-care perspective, aPDT is particularly attractive because it can be implemented using low-intensity visible light sources, such as light-emitting diodes (LEDs), and can be repeatedly activated without cumulative toxicity.⁸³ It has been shown that aPDT can concomitantly improve the immune status of the wound via neutrophil activation and release of pro-inflammatory cytokines.⁸⁰ Importantly, PSs act catalytically, enabling incorporation into or immobilisation onto polymeric dressing materials, facilitating localised, surface-mediated antimicrobial activity without continuous leaching.⁸⁴ These attributes position aPDT as a promising strategy for the treatment of infected and chronic wounds, motivating its selection as the focus of the antimicrobial approach explored in this Thesis.

2.3. Fundamentals of aPDT

The use of light for the treatment of diseases, so-called phototherapy, dates back to ancient Roman and Greek times, when heliotherapy, the use of sunlight, was practised for health maintenance.⁸⁵ A scientific basis for light-mediated

antimicrobial activity was established much later, in 1877, when Downes and Blunt demonstrated that the UV component of sunlight was lethal to bacteria.⁸⁶

2.3.1. Discovery

The photodynamic effect of dyes in the presence of light and oxygen was originally reported by medical student Oscar Raab in 1900, who observed that the protozoon *Paramecium caudatum* was more sensitive to acridine dyes after exposure to sunlight.^{45,81} Under the supervision of Hermann von Tappeiner, these findings were extended to the treatment of skin carcinoma, leading von Tappeiner to coin the term “photodynamic phenomenon”.⁸⁷ PDT requires three components: a PS, light of an appropriate wavelength, and molecular oxygen. Upon photoactivation, the PS generates ROS, which induce oxidative damage to cellular components and result in cell death.

The first therapeutic application of PDT was a combination of eosin and white light to treat superficial tumours.⁸⁸ Subsequent investigations into porphyrins as PSs, driven by their preferential accumulation in tumour tissue, led to the development of haematoporphyrin derivative (HpD) in the 1950s.⁸⁸ These advances established PDT primarily as an oncological treatment, particularly following the work of Thomas Dougherty in the 1970s, which demonstrated its efficacy in tumour therapy.^{89,90} Regulatory approval of Photofrin for the treatment of bladder cancer in the early 1990s marked a significant milestone in the clinical adoption of PDT.^{88,91}

In contrast, the application of PDT for infectious disease did not progress at the same pace. The rapid emergence and widespread use of conventional antibiotics since the 1930s has overshadowed the use of PSs for antimicrobial therapy.^{88,91} However, renewed interest in aPDT has emerged in recent decades in response to the escalating global threat of AMR.⁹¹ The ability of aPDT to induce multi-target oxidative damage via ROS generation has positioned it as a promising alternative to conventional antibiotics. Nevertheless, despite substantial preclinical data, aPDT is not yet in routine clinical practice.^{91,92}

2.3.2. Photophysical pathway

PDT relies on a sequence of well-defined photophysical and photochemical processes initiated by the absorption of light by a PS. In its ground state, the PS exists in a singlet electronic configuration, S_0 , in which all electron spins are paired and the total spin quantum number is $S = 0$. Upon absorption of a photon with sufficient energy, the PS is promoted from S_0 to an electronically excited singlet state (S_n , $n > 0$) (Figure 3). According to the Franck-Condon principle, this electronic transition occurs on a timescale that is too rapid for nuclear rearrangement, resulting in population of higher vibrational levels of the excited electronic state.

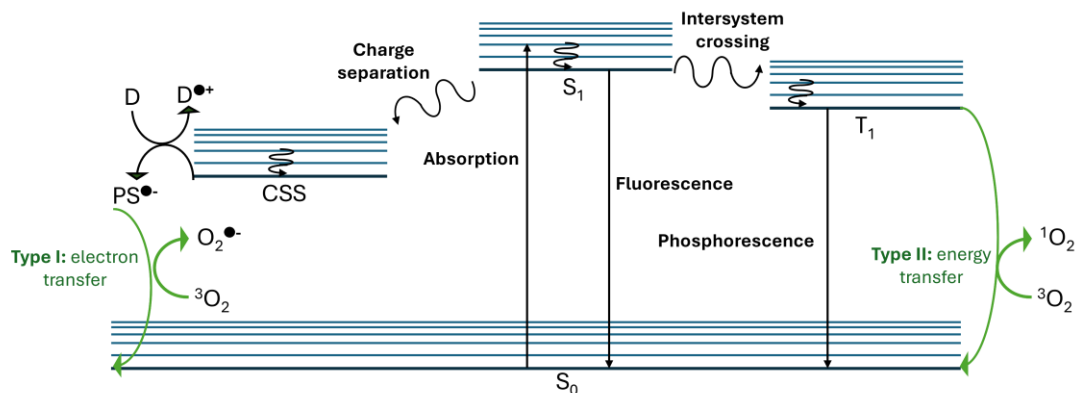


Figure 3. Jablonski diagram illustrating the Type I and Type II photochemical pathways that generate ROS in PDT. S_0 , singlet ground state; S_1 , singlet excited state; T_1 , triplet excited state; CSS, charge-separated state; PS, photosensitiser; D, electron donor.

Following excitation, rapid vibrational relaxation and internal conversion occur on the picosecond timescale, leading to relaxation to the lowest vibrational level of the first excited singlet state, S_1 .⁹³ In accordance with Kasha's rule, photophysical and photochemical processes predominantly originate from this lowest excited S_n .⁹³ The S_1 state typically has a lifetime on the order of a few nanoseconds and can decay via several competing pathways. These include radiative decay to the ground state accompanied by fluorescence emission, non-radiative decay via internal conversion, dissipating energy as heat, or intersystem crossing (ISC) to a triplet excited state, T_1 . ISC is formally spin-forbidden but can occur in many PSs due to

spin-orbit coupling, particularly in molecules containing heavy atoms or extended π -systems.

The T_1 state is characterised by a longer lifetime, typically in the microsecond range, which enables interaction with surrounding molecular oxygen ($^3O_2$) or other substrates.⁹⁴ From the T_1 state, the PS may return to the ground state via phosphorescence or non-radiative decay, or it may participate in photochemical reactions that generate ROS. There are two principal photochemical pathways: Type I and Type II.⁹⁵

In Type I photochemical reactions, the T_1 state undergoes electron transfer with nearby biomolecules or solvent molecules, resulting in the formation of radicals that react with $^3O_2$ to produce ROS such as superoxide anions, hydroxyl radicals and hydrogen peroxide, often via Fenton-like or Haber-Weiss reactions.^{95,96} In contrast, Type II photochemical reactions involve direct, spin-allowed energy transfer from T_1 to $^3O_2$, producing $^1O_2$, the electronically excited and highly reactive form of oxygen.⁹⁵

$^1O_2$ is generally considered the dominant cytotoxic agent in PDT due to its high reactivity and strong oxidising ability.⁹⁵ In $^1O_2$, electron spins in the antibonding π^* orbitals are paired, resulting in an electronically excited but diamagnetic species.⁹⁷ The lifetime of $^1O_2$ is relatively short and strongly dependent on the surrounding environment, typically ranging from microseconds to milliseconds depending on solvent polarity, temperature, and the presence of quenchers.⁹⁷ Correspondingly, its diffusion length is limited, generally on the order of 10-100 nm in biological systems, thereby spatially confining oxidative damage.⁹⁷

The high reactivity of $^1O_2$ enables it to oxidise a broad range of biological targets. It readily reacts with unsaturated membrane lipids, initiating lipid peroxidation and compromising membrane integrity. Oxidation of amino acid residues such as histidine, methionine, cysteine and tryptophan leads to protein dysfunction, while nucleic acids cause oxidative lesions that may result in mutagenic effects.^{98,99} The

efficiency of PDT is therefore strongly influenced by the photoactivation and the competing deactivation processes, such as fluorescence and phosphorescence, which reduce the quantum yield of ROS generation.¹⁰⁰

2.3.3. Mechanisms of bacterial damage

aPDT induces cell death primarily through the generation of ROS, which can inflict damage on multiple cellular targets (Figure 4). The two main mechanisms generally reported to cause lethal effects from aPDT are DNA damage and cell membrane disruption. DNA damage has been observed in the form of both single- and double-strand DNA breaks. Still, bacterial cells possess efficient DNA repair systems, suggesting that DNA damage alone is often insufficient to cause cell death.¹⁰¹ In contrast, damage to the cell membrane, through lipid peroxidation, protein oxidation or structural disruption, can lead to cell leakage or inactivation of membrane proteins, and is generally considered the primary mechanism of bacterial killing in aPDT.¹⁰¹

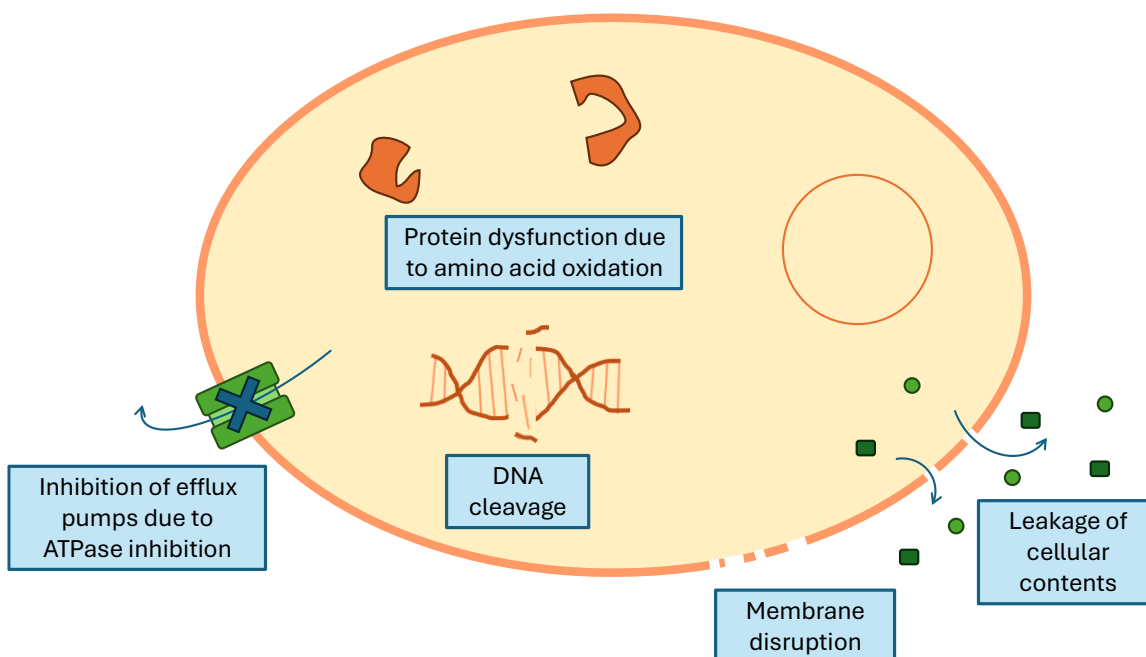


Figure 4. Mechanisms involved in the aPDT-induced inactivation of bacterial cells.

The distinct structural differences between Gram-positive and Gram-negative bacteria have profound implications for their susceptibility to aPDT. Gram-positive bacteria, with their thick but porous peptidoglycan layer and lack of outer membrane, are generally more accessible to PSs. Gram-negative bacteria, in contrast, possess an outer membrane enriched in lipopolysaccharides that can restrict the access of a PS.¹⁰² Generally, cationic PSs or co-administration of permeabilising agents are required to provide anti-Gram-negative action.¹⁰¹ Notably, studies indicate that internalisation of the PS is not strictly required for bacterial inactivation, as ROS generated at the bacterial surface are sufficiently reactive to cause cell death.¹⁰³ Interestingly, while eukaryotic cells are also susceptible to aPDT-mediated killing, some studies report the use of low light fluences to selectively kill bacteria while sparing surrounding mammalian cells.^{101,104}

It is hypothesised that, as aPDT relies on ROS generation, which acts on multiple bacterial targets simultaneously, bacteria are less likely to develop conventional resistance mechanisms.²⁵ The short PS administration-irradiation interval also precludes bacteria from recognising the otherwise non-toxic PS as the source of oxidative stress, and the extent of damage inflicted on bacterial cells may prevent cross-generational adaptivity.²⁵ This property, combined with the precise spatial and temporal control afforded by light activation, allows aPDT to provide reliable antimicrobial action while minimising off-target effects.

2.4. Photosensitisers used in antimicrobial materials

A wide range of PSs has been investigated for aPDT, encompassing different chemical classes, charges, and photophysical properties, each offering distinct advantages and limitations for wound treatment.

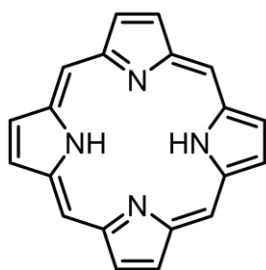
2.4.1. Classes of antimicrobial PS

PSs used in aPDT can be broadly classified into the following groups:

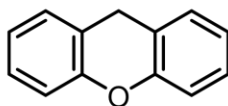
1. **Organic**, whose chemical structure can be tailored to control absorption wavelength, solubility and $^1\text{O}_2$ quantum yield.⁴³
2. **Inorganic**, where ROS are produced via light-induced electron-hole pair generation and subsequent redox reactions to yield ROS.¹⁰⁵
3. **Heavy-metal complexes**, which exploit metal-centred photophysics to enhance ISC and ROS generation.¹⁰⁶

Among these categories, in contrast to inorganic PSs or heavy-metal complexes, which may raise toxicity concerns, organic PSs are generally less cytotoxic.¹⁰⁶ Organic PSs also provide tunable chemical structures and ease of incorporation into carrier materials, making them particularly well-suited for wound dressing formulations.¹⁰⁷ The main chemical classes of organic PSs (Scheme 1) are:

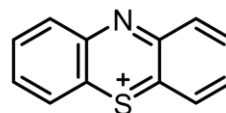
1. **Porphyrin** derivatives, which are frequently used in oncology and increasingly explored for bacterial inactivation.¹⁰⁸
2. **Xanthene** derivatives, including eosin Y, Rose Bengal and Phloxine B, with enhanced ISC and ROS generation and are generally water-soluble.¹⁰⁹
3. **Phenothiazinium** derivatives, such as methylene blue and toluidine blue O, which are cationic and can electrostatically interact with bacterial membranes.¹¹⁰



porphyrin



xanthene



phenothiazinium

Scheme 1. Molecular structures of the main chemical classes of PSs.

2.4.2. Selection criteria

The choice of a PS for incorporation into wound dressings requires careful consideration of several properties, including:

- ROS quantum yield: the ideal PS would be highly phototoxic with a high ROS quantum yield.²⁵
- Photostability: resistance to photodegradation ensures sustained activity during light exposure.
- Cytocompatibility: the PS should have minimal dark toxicity.²⁵
- Absorption wavelength: generally, the penetration of the required wavelength of light to the desired area is a consideration, with red and near-infrared absorption preferable for deeper tissues.¹¹¹ In superficial infections, wavelengths in the visible range are sufficient.
- Regulatory acceptance: PSs already approved for clinical use also provide translational advantages.

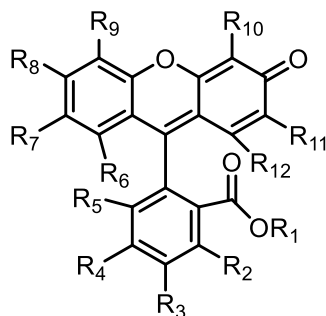
2.4.3. Advantages of xanthene dyes for incorporation into wound dressings

Many xanthene-based dyes are particularly well-suited for aPDT applications due to their water solubility, anionic charge and high ROS generation efficiency. Many fluorescein derivatives are ideal for incorporation into polymeric carriers because they contain a free carboxylate group. Moreover, many xanthene derivatives are FDA-approved as colouring agents for cosmetics, food, and drugs.^{112,113}

2.4.4. Phloxine B as a model PS

Phloxine B (3',4',5',6'-tetrachloro-2,4,5,7-tetrabromofluorescein, PhB) is an acidic, halogenated, less fluorescent derivative of fluorescein (Scheme 2). It is FDA approved (FDA code Red No. 28) as a food and cosmetic colouring.¹¹³ As an anionic PS, it is generally associated with activity against Gram-positive bacteria.¹¹⁴

However, extracellular accumulation of PSs has also been reported to facilitate ROS-mediated damage to Gram-negative bacteria, especially when bacterial membrane-stabilising cations Ca^{2+} and Mg^{2+} are displaced.^{25,115}



Scheme 2. General molecular structure of fluorescein derivatives. For PhB, $\text{R}_1=\text{Na}^+$, $\text{R}_{2-5}=\text{Cl}$, $\text{R}_{6\&12}=\text{H}$, $\text{R}_{7,9-11}=\text{Br}$, $\text{R}_8=\text{O}^-\text{Na}^+$.

PhB was first reported in 1926 as a bacterial staining agent, and it has since been used in cytological stains due to its intense colouration and reduced fluorescence compared to fluorescein.¹¹⁶ The incorporation of bromine atoms increases spin-orbit coupling, promoting ISC from S_1 to T_1 and stabilising the triplet state, which is critical for ROS generation. Its singlet oxygen quantum yield, Φ_Δ , is reported to be 0.40.¹¹⁷

A limitation of xanthene derivatives, including PhB, is their photostability, as ROS produced during irradiation can oxidise the dye itself.^{118,119} Strategies to improve stability include reducing photon absorption and inhibiting energy transfer.¹¹⁸

2.5. Polymeric platforms for antimicrobial photodynamic materials

The practical application of PSs in wound care requires the incorporation into functional materials. Many PSs suffer from limitations, including poor aqueous solubility, aggregation, photobleaching, and non-selective cytotoxicity towards host tissues.¹²⁰ These challenges have therefore led to the research field of so-called third-generation PSs, which incorporate PSs into delivery vehicles to enhance

stability, localisation, and maximise antimicrobial efficacy while minimising off-target effects.¹²¹ While free PSs may diffuse, reducing in concentration, encapsulated or immobilised PSs result in high ROS concentrations at the bacteria-material interface.

Polymeric materials offer a versatile platform for developing aPDT coatings, owing to their chemical tunability, processability, and established biocompatibility in wound care applications.¹²² Their ability to be fabricated into diverse formats, such as thin films, hydrogels, fibres or nanoscale assemblies, enables precise control over photosensitiser localisation and interfacial interactions with bacteria. The $^1\text{O}_2$ generation efficiency of PhB in combination with a polymeric system was first investigated by Litman et al. in 2016, where they loaded PhB into poly(2-hydroxyethyl methacrylate) (PHEMA) thin films by spin coating solutions of PHEMA and PhB, providing a proof of concept for the use of PhB in photoactive coatings.¹²³ Subsequently, in 2021, Dadi et al. loaded poly(urethane) (PU)-based films with PhB by solution mixing.¹²⁴ Laser irradiation afforded significant anti-biofilm activity against *S. aureus*, indicating that PhB incorporated on a surface remains active.¹²⁴ However, as both systems rely on non-specific loading of PhB, they may lead to dye leaching, reducing efficacy over time. More recently, in 2025, Ybarra et al. reported the self-assembly of nanoparticles from polyamidoamine dendrimers loaded with PhB, which were shown to exhibit antibacterial activity against both *S. aureus* and *P. aeruginosa* upon irradiation, however, no mechanistic basis for successful PhB delivery was discussed.¹²⁵ There is therefore a lack of literature on the requirement for PhB internalisation for successful aPDT activity.

2.5.1. Nanocarriers in third-generation PSs

The use of nanoparticles composed of lipids, polymers or inorganic materials has been explored as potential PS carriers.³² Among these, polymer-based nanocarriers are particularly attractive due to their structural diversity and compatibility with soft tissue applications.¹²⁶ Polymeric micelles and vesicles formed from amphiphilic

block copolymers enable compartmentalisation of PSs.¹²⁶ The benefits of encapsulating PSs in polymers have been demonstrated, for example, by Brival et al., who encapsulated pheophorbide α (Pheo) in poly(ethylene oxide)-*block*-poly(ϵ -caprolactone) (PEO-*b*-PCL) micelles, and found enhanced intracellular penetration by 6.8-fold compared to free Pheo in human colorectal cancer cells.³² Subsequent light activation induced membrane protrusions characteristic of apoptosis, indicating successful delivery of the Pheo.³²

Further interesting work by Taguchi et al. in 2025 showed the self-assembly of micelles from a monomer with the PS phthalocyanine covalently bonded.¹²⁷ These micelles generated $^1\text{O}_2$ and exhibited slight photocytotoxicity against human breast cancer cells, further indicating that PS internalisation is not necessary for effective PDT.¹²⁷

2.5.2. Functional polymers in third-generation PSs

Beyond encapsulation, polymers can actively enhance PDT efficacy. Conjugated polymers, for example, have been employed to enhance the PDT effects of inorganic PSs through their large, delocalised electronic structures, which facilitate exciton migration along the polymer backbone. This enables efficient energy transfer to nearby PSs, promoting singlet oxygen generation under both one- and two-photon excitations.¹²⁸

Hybrid polymers incorporating nanostructures have also been explored. For example, carbon quantum dots (QDs) were incorporated into PU films by swelling in a QD solution, yielding nanocomposites with no observable leaching after 24 hours in water.¹²⁹ Upon irradiation (6 h), these materials exhibited up to 5 log₁₀ reductions in *S. aureus* and *E. coli* CFUs compared to control PU films.¹²⁹ Such systems demonstrate how polymers can facilitate the activation of PSs.

2.6. Amphiphilic block copolymers for self-assembly of polymeric carriers

Amphiphilic block copolymers (ABCPs) consist of two or more chemically distinct polymer blocks covalently bonded. Due to the thermodynamic incompatibility between the blocks, ABCPs undergo microphase separation, leading to the spontaneous formation of ordered nanostructures in both bulk and solution.¹³⁰ This self-assembly behaviour enables their use in nanotechnology and drug delivery.³⁷ While PS-loaded ABCP assemblies have been extensively investigated for anticancer applications, with a focus on improved PS solubility and circulation time, their application in aPDT remains comparatively limited.^{131–134} There is therefore a need for further exploration of ABCP-based PS carriers specifically for antibacterial applications.

2.6.1. Phase separation in bulk

In the bulk, ABCP self-assembly is governed by the thermodynamics of microphase separation, first described by Leibler in 1980.¹³⁵ This phase behaviour is primarily dictated by the product χN , where χ is the Flory-Huggins interaction parameter, which describes the incompatibility of the blocks, while N is the total degree of polymerisation. When χN exceeds a critical value, the system undergoes an order-disorder transition and is governed by enthalpic terms, forming defined microdomains.¹³⁰ The equilibrium morphology depends on the volume fraction (f) of each block, resulting in structures such as lamellae, cylinders, gyroids and spheres, which have been extensively validated experimentally using small-angle X-ray scattering (SAXS) and small-angle neutron scattering (SANS).¹³⁰

2.6.2. Self-assembly in solution

In selective solvents, ABCP self-assembly is driven by the minimisation of interfacial free energy between solvophobic blocks and the solvent, while solvophilic blocks stabilise the assembly.¹³⁰ This process is analogous to surfactant

micellisation; above a critical micelle concentration (CMC), ABCPs self-assemble into nanoscale structures.¹³⁰ Compared to surfactant assemblies, polymeric structures generally exhibit enhanced kinetic stability due to their higher molecular weight.

Poly(styrene)-*block*-poly(acrylic acid) block copolymers have been extensively studied.^{136–138} In 1995, Zhang and Eisenberg reported, for the first time, the observation of 6 stable morphologies by varying the hydrophilic block length while keeping the hydrophobic block length fixed.¹³⁶ It is now known that the formation of thermodynamically stable morphology depends on the degree of stretching of the core-forming blocks, the interfacial tension between the core and the solvent and the repulsive interactions among corona-forming chains.¹³⁹ Practically, to direct the self-assembly towards a particular morphology, the copolymer composition and concentration, antisolvent content, and the nature of the common solvent can be varied.¹³⁰

The so-called packing parameter (p , Equation 1) is used to predict the architecture of the resulting assembly, and depends on the volume, v , and length, l , of the solvophobic block, and the effective interfacial area of the solvophilic block, a_0 . When $p < 0.3$, micellar structures are formed, then cylindrical structures when $0.3 < p \leq 0.5$, and vesicular structures when $0.5 < p \leq 1$.^{130,140} The resulting morphology determines the functional properties of the assembly, for example, the loading capacity and diffusivity, parameters directly relevant to applications in aPDT.

$$p = \frac{v}{a_0 l} \quad \text{Equation 1}$$

2.6.3. Block copolymer assemblies for antimicrobial delivery

Self-assembled ABCP nanostructures enable encapsulation of small molecules.³⁷ Since their proposal as drug carriers by Ringsdorf in 1984, PEG-based ABCP assemblies have become a cornerstone of nanomedicine due to their

biocompatibility and so-called stealth properties.³⁷ While this has been demonstrated extensively with dye molecules and conventional antimicrobials, the encapsulation of PSs in such systems has received limited interest.^{127,141} In terms of aPDT in wounds, encapsulation provides a strategy to localise the PS at the target site and minimise loss of the active compound due to diffusion.

2.7. Biodegradable amphiphilic block copolymers

ABCPs have been synthesised from a wide range of monomers. The introduction of stimuli-responsive blocks can result in functional block copolymers responsive to a range of stimuli, including pH, temperature or light.¹⁴¹ The use of biodegradable polymers is particularly advantageous for use in medicine. Polyesters such as poly(lactic acid) (PLA), poly(ϵ -caprolactone) (PCL) and poly(glycolic acid) (PGA) degrade primarily through hydrolytic cleavage of ester bonds and are widely used in biomedical applications owing to their low cost and FDA approval.^{141,76} Although other polymers, such as poly(anhydrides), poly(phosphazenes) and poly(cyanoacrylates), are also degradable, concerns over the toxicity of their degradation products limit their clinical relevance.³⁸

Synthesis of such polymers can be achieved via various methods. Conventionally, free radical polymerisation was used, resulting in polydisperse products. Controlled radical polymerisations, such as atom transfer radical polymerisation (ATRP) or reversible addition-fragmentation chain transfer (RAFT) polymerisation, lead to better-defined architectures and reduced polydispersity.¹⁴¹

2.7.1. Ring-opening polymerisation

Ring-opening polymerisation (ROP) is one of the most widely used synthetic strategies for preparing polyesters from cyclic monomers.¹⁴² Its prominence arises from the ability of ROP to afford polymers with predictable molar mass, narrow dispersity and well-defined end-group functionality, features that are difficult to

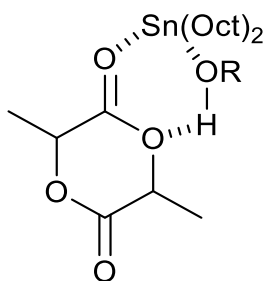
achieve using conventional step-growth polycondensation. These attributes make ROP particularly attractive for the synthesis of polyesters.

The mechanism of ROP is dependent on the nature of the initiator and catalyst used. Alkoxide bases (e.g. KOtBu) and organometallic reagents (e.g. *n*-BuLi, CH₃MgBr) typically promote anionic ROP, while protic acids (e.g. H₂SO₄) or Lewis acids (e.g. AlCl₃) result in cationic ROP via an active chain-end mechanism. In contrast, Lewis bases such as pyridine and ionic catalysts, including *N*-heterocyclic carbenes, operate through an activated monomer mechanism, in which the monomer is directly activated without generating charged propagating chain ends, resulting in fewer side reactions such as inter- and intramolecular transesterification, often found in anionic ROP and leading to broad molar mass distributions.¹⁴²

Metal-catalysed ROP typically proceeds via a coordination-insertion mechanism, which has become the dominant method for the controlled polymerisation of lactones and lactides.¹⁴³ Among these catalysts, tin(II) octoate (Sn(Oct)₂) remains the most widely used, particularly in industrial and biomedical contexts, as it is FDA-approved.¹⁴³ In this mechanism, Sn(Oct)₂ acts as a Lewis acid, coordinating with the carbonyl oxygen of the cyclic ester and thereby activating the carbonyl group.¹⁴⁴ The nucleophilic hydroxyl terminus of an alcohol initiator, such as PEG methyl ether (mPEG), attacks the activated carbonyl carbon, inducing ring opening and forming an alkoxide-terminated growing chain. Subsequent coordination and insertion of additional monomer units result in chain propagation.

When protic initiators such as alcohols, amines or thiols are employed, Sn(Oct)₂ can coordinate simultaneously to both the monomer and the initiator (Scheme 3).^{145,146} This dual coordination pathway renders the polymerisation sensitive to moisture, as adventitious water can act as a competing initiator and compromise control over molar mass.¹⁴⁵ Nevertheless, due to the low polymerisation enthalpy of lactides ($|\Delta H| = 29$ kJ/mol), controlled ROP can be achieved in the bulk, without the

need for solvent, which is advantageous from both economic and environmental perspectives.¹⁴⁵



Scheme 3. Coordination-insertion complex formed for $\text{Sn}(\text{Oct})_2$ -catalysed ROP in the presence of a protic initiator.

Beyond tin-based catalysts, a wide range of alternative metal-containing systems have been investigated for the ROP of lactones, including zinc or aluminium alkoxides, as well as rare-earth metal complexes, which have attracted interest due to their enhanced activity, improved stereocontrol and, in some cases, reduced toxicity.¹⁴⁷ Such catalyst systems provide additional opportunities in applications where residual metal content is a concern.

2.8. Surface-bound and immobilised PS-polymer systems

While ABCP self-assembly enables access to nanoscale carriers for PSs, they remain mobile systems and therefore the antimicrobial activity depends on the diffusion, retention and clearance from the wound environment. In contrast, the immobilisation of PSs onto polymeric surfaces represents a powerful strategy for localised aPDT, where ROS are generated directly at the material-bacteria interface, enabling contact-mediated killing without systemic exposure.¹⁴⁸ Immobilised PS systems have been demonstrated to reduce PS leaching and off-target toxicity, localised and spatially controlled antimicrobial action, reusability and prolonged lifetime and compatibility with existing wound dressing materials.⁸⁴ Covalent coupling of the xanthene derivative Rose Bengal to polystyrene-based beads via nucleophilic displacement was demonstrated in an attempt by Schaap et al. to create polymer-based PS systems.¹⁴⁹ These systems were confirmed to retain

antimicrobial activity upon irradiation by Bezman et al. in 1978, indicating the possibility of PS-polymer conjugates for successful aPDT applications.¹⁵⁰

2.8.1. Hydrogels

Hydrogels, crosslinked 3D structured networks of hydrophilic polymer chains capable of absorbing water, are widely regarded as possessing many characteristics of an ideal wound dressing, as they maintain a moist wound environment, provide cooling and are often non-irritating.^{71,151} PSs can be incorporated into hydrogels through either physical entrapment or covalent attachment.^{152,153} While physical entrapment may enable higher loading, such systems are particularly susceptible to leaching.¹⁵³ On the other hand, covalent immobilisation generally offers superior stability and localisation, providing a more suitable option for sustained aPDT.¹⁵²

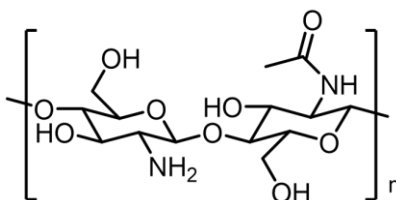
2.8.2. Thin films and coatings

In photodynamic coatings, the localisation of the PS within the film directly influences the antimicrobial efficacy due to the limited diffusion distance of ROS.¹⁵⁴ Hence, thin polymeric films provide an opportunity for enhanced aPDT activity relative to volume. Many polymers are capable of forming films.¹⁵⁵ Early wound dressings based on nylon films provided mechanical protection but exhibited limited capacity for wound exudate absorption, leading to fluid accumulation, skin maceration and an increased risk of infection.⁷¹ More advanced materials, such as PU-based films including Opsite™, offer improved moisture permeability, transparency and elasticity.⁷¹ While synthetic polymers have demonstrated the feasibility of surface-immobilised aPDT, increasing attention has shifted toward biopolymer-based coatings due to their inherent biocompatibility, making them particularly attractive for wound dressing applications.¹⁵⁴

2.8.3. Chitosan as a polymeric coating for wound dressings

Biomaterials used in wound dressings can play an active role in both wound healing and infection control.⁷¹ Beyond providing mechanical protection, modern wound dressings are increasingly designed to modulate the wound microenvironment, regulate moisture and prevent or eradicate microbial contamination.⁷⁴ A range of natural polymers, including collagen, alginate, hyaluronic acid and chitosan (CS), have been explored as potential wound dressings.⁷¹ Among these materials, CS has emerged as a particularly attractive platform due to its biocompatibility, intrinsic antimicrobial activity and chemical versatility, making it well-suited for integration with immobilised photodynamic systems.¹⁵⁶

CS is the partially deacetylated derivative of chitin, a linear polysaccharide composed of glucosamine and *N*-acetyl-glucosamine.¹⁵⁶ One of the most abundant biopolymers, chitin can be isolated from crustacean cells, insect cuticles, algae and fungal cell walls.¹⁵⁶ Industrial extraction typically involves alkaline treatment to remove proteins, acid washing to eliminate mineral contaminants, oxidative decolourisation and subsequent deacetylation under highly alkaline conditions to yield CS.¹⁵⁷



Scheme 4. Molecular structure of CS at pH > pKa.

The physicochemical properties of CS depend on its molecular weight and degree of deacetylation, which govern its solubility, charge density and biological activity.¹⁵⁶ As the only naturally occurring polycation, CS possesses a high density of primary amines, which are protonated under mildly acidic conditions.¹⁵⁶ When in solution, these protonated amines electrostatically interact with negative bacterial cell surfaces, permeabilising the bacterial cells, leading to cell death.^{157,158} In

addition, primary and secondary hydroxyl groups provide further reactive sites for chemical modification. CS is widely recognised for its biocompatibility and low toxicity.¹⁵⁷ It has been approved as an excipient by the FDA and has been extensively investigated for biomedical applications as films, gels, nanofibers and nanoparticles, providing flexibility in material design.¹⁵⁷

3. Objectives of this Thesis

While PDT is clinically established for oncological applications, translation of aPDT requires improved PS stability, localisation and reduced diffusion to overcome the inherent limitations of solution-based PS delivery. Polymers have been extensively explored both as nanoscale delivery vehicles, enabling encapsulation of drugs in self-assembled structures, and as macroscopic substrates, allowing immobilisation of drugs onto surfaces. The aim of this Thesis is therefore to develop and evaluate polymer-based photodynamic systems for the treatment of wound-associated bacterial infections. Specifically, the work focused on:

1. The investigation of PhB as an FDA-approved dye for the aPDT of *S. aureus* and *P. aeruginosa*.
2. The synthesis, characterisation and self-assembly of ABCPs to form PhB-loaded vesicles and assess their applicability as aPDT agents.
3. The development of a PhB-functionalised biopolymer film for contact-based antimicrobial activity against planktonic bacteria and biofilms.

These systems were developed to determine whether PhB internalisation is necessary for effective aPDT, or whether surface-bound systems can provide comparable antimicrobial activity.

4. Photophysical characterisation and antimicrobial activity of Phloxine B

4.1. Introduction

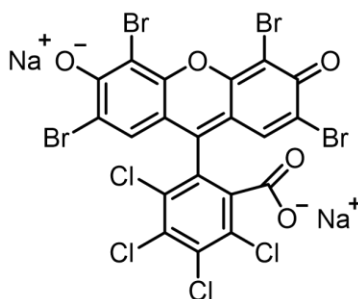
The intrinsic photophysical properties, stability, and biological activity of the PS itself ultimately constrain the performance of polymer-based photodynamic systems. Understanding of these baseline characteristics is therefore a prerequisite to evaluating any delivery or immobilisation strategies. PhB was selected as the model PS in this Thesis owing to its strong absorption of green light, leading to efficient $^1\text{O}_2$ generation.^{159,160} Importantly, PhB is a low-cost dye with an established safety profile, making it attractive for translation into wound care applications.¹¹³ However, despite its frequent use in aPDT studies, systematic evaluation of its use as a PS remains limited.^{160–162}

This Chapter, therefore, investigates the photophysical properties, photostability, and baseline aPDT of free PhB in solution. By identifying the capabilities and limitations of free PhB, this Chapter provides the necessary context for the vesicle-based and surface-immobilised systems investigated in Chapters 5 & 6.

4.2. Photophysical properties of PhB

4.2.1. Absorption and emission spectra

PhB is a halogenated xanthene derivative whose photophysical behaviour is dominated by π - π^* transitions within the conjugated xanthene core (Scheme 5).⁹⁴ The extended aromatic system gives rise to intense visible-light absorption, while heavy halogen substitution significantly influences excited-state deactivation pathways.⁹⁴



Scheme 5. Molecular structure of Phloxine B.

In aqueous solution, PhB exhibits a strong absorption band centred at 539 nm, corresponding to the singlet ground state to singlet excited state ($S_0 \rightarrow S_1$) electronic transition (Figure 5a).⁹⁴ A weaker vibronic shoulder is visible at 504 nm, which arises from coupling between the electronic and vibrational transitions. Below a concentration of 50 μM , the absorbance at the maximum wavelength decreases linearly with concentration, indicating compliance with the Beer-Lambert law and the absence of aggregation effects within this range (Figure 5b). This high molar absorptivity in the green region enables efficient excitation using low-cost, low-power light sources, a key advantage for translating PhB-mediated aPDT applications to the clinic.

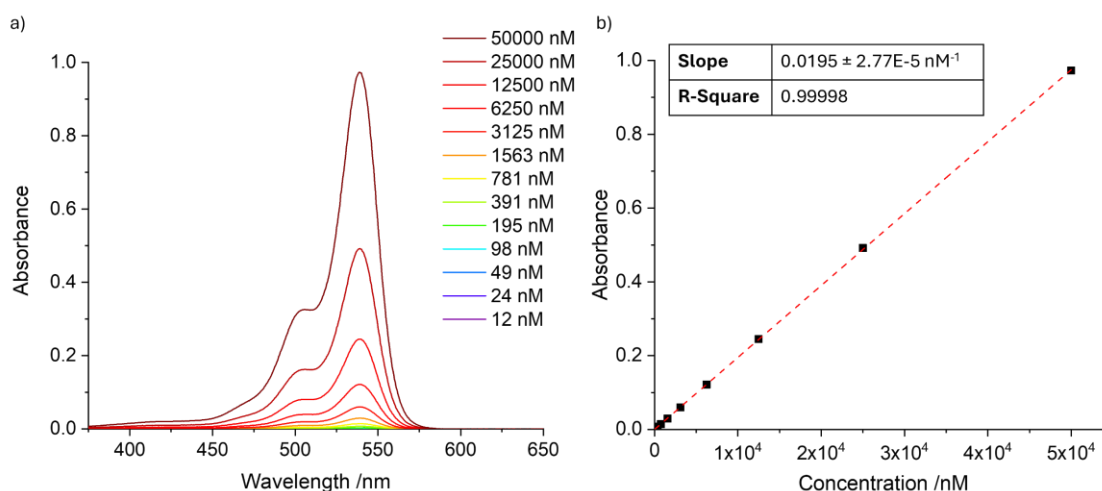


Figure 5. a) Absorption spectra of PhB in Milli-Q water and b) corresponding calibration curve of absorbance at the absorption maximum as a function of PhB concentration.

Upon excitation, PhB displays fluorescence emission in the yellow region of the visible spectrum, typically with an emission maximum around 565-580 nm (Figure 6a). The relatively small Stokes shift is characteristic of xanthene dyes and reflects limited reorganisation between the ground and first excited singlet state.¹⁶³ The fluorescence intensity increases linearly with concentration over the investigated range, indicating minimal self-quenching below 5 μM (Figure 6b).

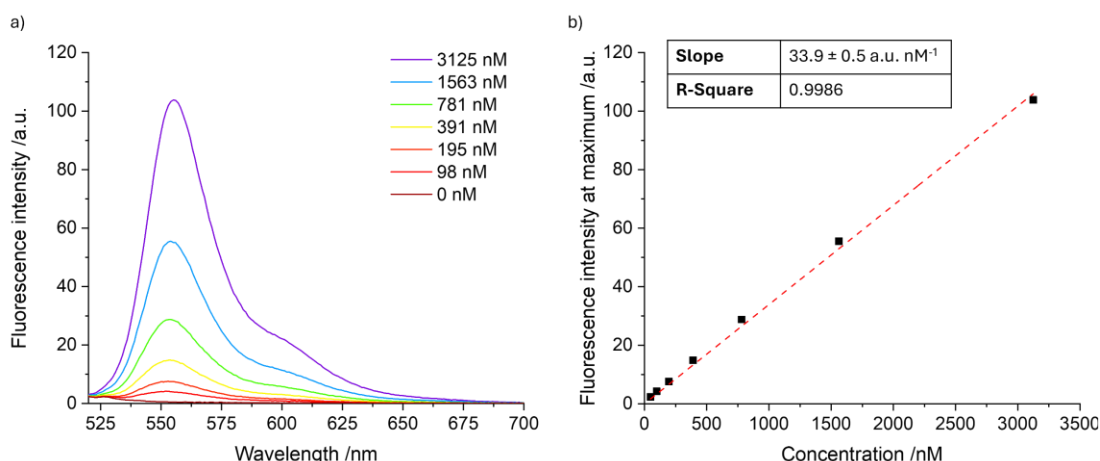
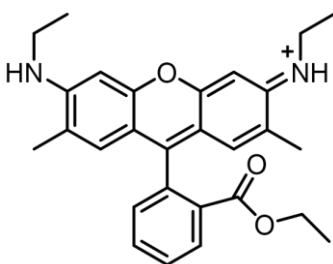


Figure 6. a) Fluorescence emission spectra of PhB in Milli-Q water and b) associated calibration curve of fluorescence emission intensity at maximum emission wavelength vs PhB concentration ($\lambda_{\text{ex}} = 504 \text{ nm}$).

4.2.2. Fluorescence quantum yield

The fluorescence quantum yield (ϕ_F) of a fluorophore quantifies the efficiency with which absorbed photons are converted into fluorescence emission and is defined as the ratio of the number of photons emitted to the number absorbed. For xanthene dyes, ϕ_F is strongly influenced by the presence of heavy atoms that modulate the excited-state deactivation pathway. The ϕ_F of PhB is reported to be moderate compared to that of non-halogenated xanthene analogues such as fluorescein.¹⁶⁴ This reduced fluorescence efficiency is primarily due to the heavy atom effect imparted by the bromine substituents.¹⁶⁴ These heavy atoms enhance spin-orbit coupling, thereby promoting ISC from S_1 to T_1 .¹⁶⁵ This enhanced T_1 population is a critical prerequisite for aPDT, as it facilitates subsequent energy transfer to ground-state molecular oxygen, thereby generating $^1\text{O}_2$.¹⁶⁶

The ϕ_F of PhB in water was determined here using a relative method with rhodamine 6G (R6G, Scheme 6) as the reference standard. R6G was selected due to its high and well-characterised ϕ_F (0.95 in water).¹⁶⁷ Although absolute ϕ_F measurements are preferable, relative measurements provide a reliable and experimentally accessible means of comparison when performed against a suitable reference.



Scheme 6. Molecular structure of Rhodamine 6G.

The ϕ_F of PhB was calculated from the integrated fluorescence intensities (I) of PhB and R6G, corrected for differences in absorbance at the excitation wavelength (A) and the refractive indices of the solvents (n), according to Equation 2. By matching the absorbance of the PhB and R6G solutions at the excitation wavelength and using the same solvent, the equation simplifies to Equation 3.

$$\phi_F^{PhB} = \phi_F^{R6G} \cdot \frac{I_{PhB}}{I_{R6G}} \cdot \frac{A_{R6G}}{A_{PhB}} \cdot \frac{n_{PhB}^2}{n_{R6G}^2} \quad \text{Equation 2}$$

$$\phi_F^{PhB} = \phi_F^{R6G} \cdot \frac{I_{PhB}}{I_{R6G}} \quad \text{Equation 3}$$

Excitation at 504 nm enables clear spectral separation between excitation and emission bands, facilitating reliable integration of the emission signal. The integrated emission intensity of PhB was 2421 a.u. nm, corresponding to 36% of the integrated emission intensity of R6G (6367 a.u. nm) under identical conditions (Figure 7). According to Equation 3, this corresponds to a ϕ_F for PhB in water of 0.36. Literature reports indicate ϕ_F of ~0.76 for PhB in ethanol, with more moderate ϕ_F expected in other environments, due to a strong dependence on the dye concentration and microenvironment.¹²³ Relative to R6G, the heavy-atom effect

associated with the bromine substitution is well established to reduce the ϕ_F of halogenated xanthene dyes relative to their non-halogenated analogues.¹⁶⁸ The ϕ_F for PhB determined here is therefore consistent with literature expectations for PhB under aqueous conditions. As a lower ϕ_F is indicative of enhanced non-radiative decay via ISC and therefore higher probability of T_1 formation, this indicates a greater likelihood of $^1O_2$ generation. In line with this photophysical behaviour, PhB reportedly exhibits a relatively high $^1O_2$ quantum yield (ϕ_Δ), reported to be ~ 0.40 in ethanol, supporting its use as an effective PS for aPDT.^{160,169}

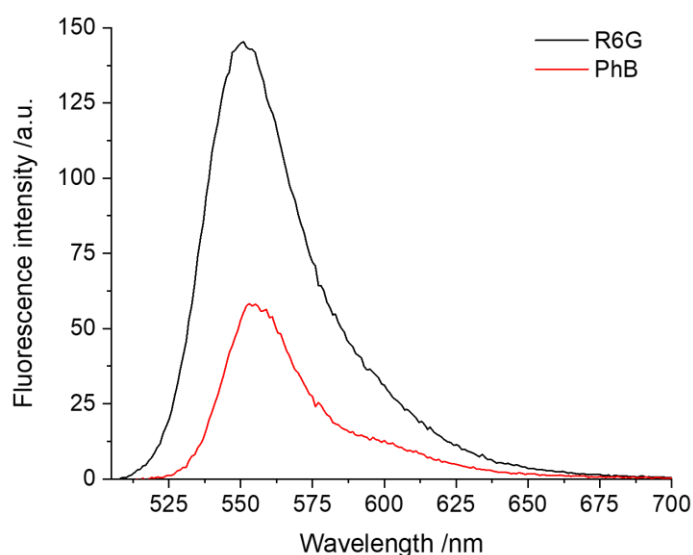


Figure 7. Fluorescence emission spectra of aqueous R6G and PhB solutions ($\lambda_{ex} = 504$ nm).

4.2.3. pH-dependent absorption behaviour of PhB

While ϕ_F for ISC govern the fate of the excited state, the population of these excited states is dictated by the ground state electronic structure and absorption properties of the PS.¹⁶⁵ For xanthene dyes such as PhB, this structure, and therefore the absorption spectrum, are highly sensitive to protonation state and therefore strongly dependent on pH.

The pH-dependent absorption of PhB was therefore investigated by adjusting the pH of a PhB solution prepared in Britton Robinson buffer. This buffer system was selected to enable tuning of pH over a wide range while maintaining constant ionic

strength, thereby minimising secondary effects on dye aggregation or spectral broadening. Absorption spectra were first recorded at pH 1.1, followed by incremental increases in pH. At low pH, protonation results in PhB predominantly adopting a spirolactone formation, in which conjugation across the xanthene core is disrupted.¹⁷⁰ This reduced delocalisation increases the HOMO-LUMO energy gap and results in minimal absorption in the UV-Vis spectrum (Figure 8). As pH increases, deprotonation shifts the equilibrium towards the quinoid form, then the open phenolate form.¹⁷⁰ These structures exhibit extended π -conjugation across the xanthene core, lowering the HOMO-LUMO energy gap and increasing the absorption of visible light.

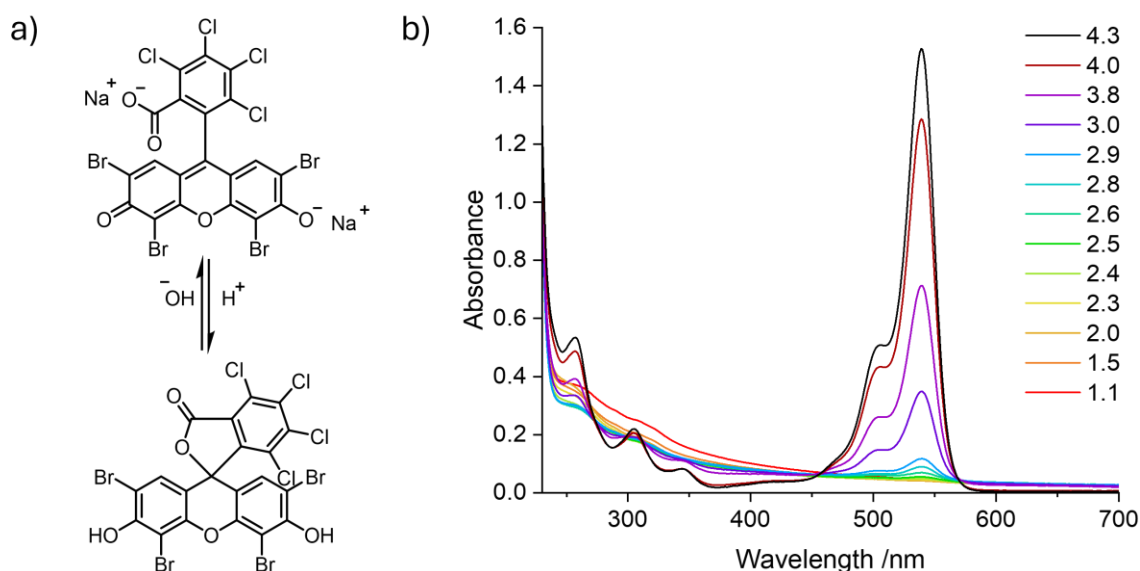


Figure 8. a) pH-dependent structural transformation of PhB between its deprotonated (basic) and protonated (acidic) form, visible in b) the change in absorption of PhB upon increasing pH.

This pH-dependent behaviour is well-documented for other xanthene dyes such as erythrosine and Rose Bengal.¹⁷¹ Importantly, only the deprotonated, conjugated forms of PhB exhibit absorption bands capable of efficient excitation to the S_1 state. Consequently, aPDT is strongly suppressed under acidic conditions, where excitation and ISC are limited. For use in aPDT, it is therefore important to operate within pH regimes that favour the open, conjugated forms of PhB, facilitating

efficient excitation to the S_1 state, leading to enhanced ISC to T_1 , and, consequently, more energy transfer to $^3O_2$ to generate $^1O_2$.

4.3. Photostability of PhB under irradiation

While favourable absorption and T_1 population are prerequisites for efficient aPDT, the practical utility of a PS is ultimately constrained by its photostability under irradiation. Photobleaching reduces the effective concentration of an active PS during treatment and can limit both treatment efficacy and reproducibility. Xanthene dyes are known to undergo self-sensitised photodegradation due to their efficient generation of $^1O_2$. The photostability of PhB was therefore evaluated under extreme irradiation conditions by irradiating an aqueous PhB solution prepared in PBS adjusted to an $OD_{539} = 1.0$ in a sealed 1 cm quartz cuvette. The initial absorption spectrum was recorded prior to irradiation, after which the sample was exposed to continuous illumination from a 575 W mercury halide lamp. Absorption spectra were subsequently collected at 30-minute intervals over a 4-hour period, then again after 24 hours of irradiation (Figure 9).

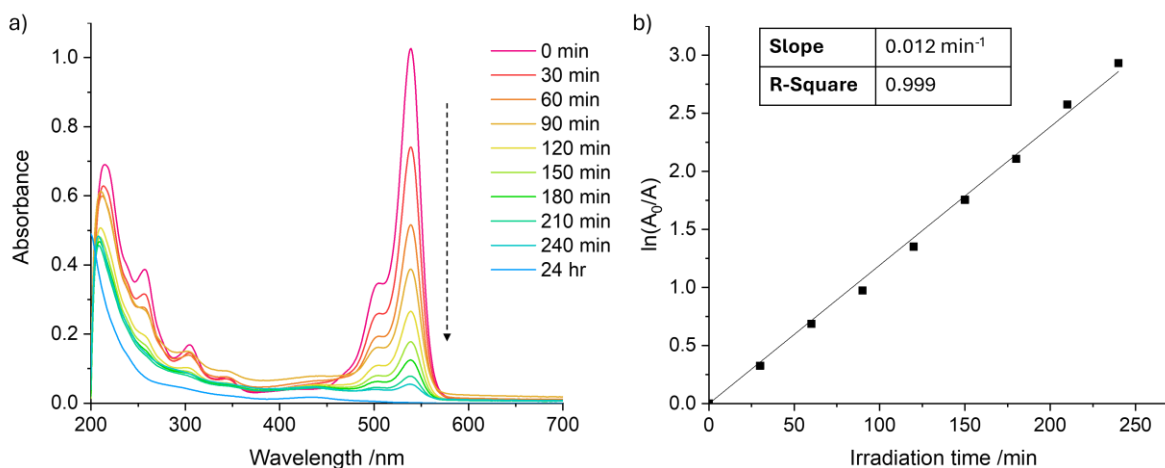


Figure 9. Photodegradation of PhB upon exposure to a 575 W mercury halide lamp, represented a) as absorption spectra, and b) in a pseudo-first order kinetic plot.

Under these conditions, PhB was found to be photolabile, exhibiting a progressive decrease in absorbance at 539 nm with irradiation time, with complete depletion of

this characteristic absorption band after 24 hours. No significant spectral shifts or new absorption features were observed during degradation, indicating loss of conjugation rather than formation of stable photoproducts with distinct electronic transitions. This behaviour is consistent with photobleaching mechanisms reported for other halogenated xanthene dyes.^{172,173}

The photodegradation kinetics were analysed by fitting the decrease in absorbance at 539 nm to a pseudo-first order rate model, which is typical for xanthene derivatives.^{172,173} A degradation rate constant of 0.012 min^{-1} was obtained, corresponding to a photobleaching half-life of ~58 min under these conditions. For comparison, a previous study using a lower-power 150 W metal halide lamp found a degradation rate of 0.0019 min^{-1} .¹⁷³ The substantially faster degradation observed here, representing a 6.3× increase in rate despite only a 3.8× increase in lamp wattage, could be attributed to differences in the spectral output of the light sources or to additional thermal effects associated with the higher-wattage lamp, leading to faster degradation. Photobleaching of xanthene dyes is widely attributed to self-sensitised oxidation of the aromatic rings mediated by $^1\text{O}_2$, in which $^1\text{O}_2$ attack disrupts π -conjugation. This is evident in the spectra as no significant shifts or new peaks appear upon degradation, suggesting irreversible chemical degradation of the chromophore.¹⁷³

4.4. Baseline aPDT of free PhB

Following characterisation of the photophysical properties, pH-dependent absorption behaviour and photostability of PhB, its baseline aPDT was evaluated under conditions relevant to *in vitro* biological assays to establish the intrinsic aPDT efficacy of free PhB.

4.4.1. Excitation with green LEDs

Irradiation experiments were performed using an in-house-built LED irradiation setup designed to deliver uniform green-light excitation across multiwell plates.

Green light-emitting diodes (LEDs) were selected to provide spectral overlap with the absorption bands of PhB while minimising unnecessary exposure at shorter wavelengths. The emission spectrum of the LEDs spans approximately 450 - 570 nm, with a maximum intensity centred at ~520 nm (Figure 10), overlapping well with the PhB absorption.

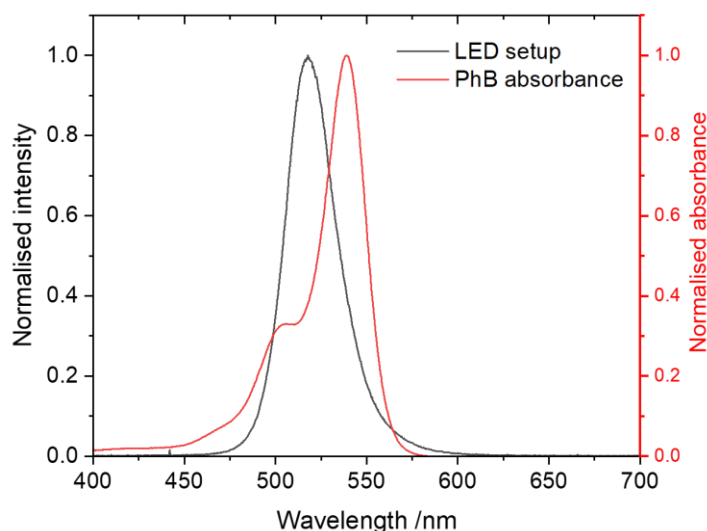


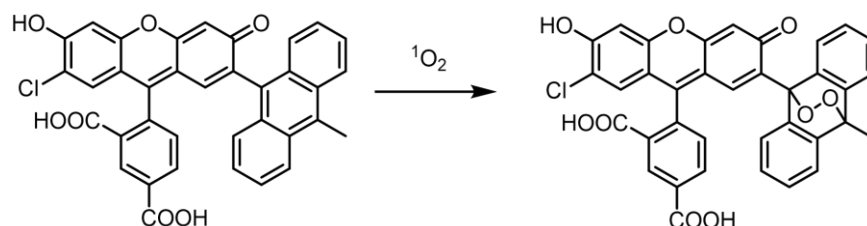
Figure 10. Intensity of green LED array overlayed with the absorption spectrum of PhB in water.

The LED array consisted of 96 individual green LEDs aligned with the wells of a standard 96-well plate, enabling parallel irradiation of all samples under identical conditions. The irradiation intensity was measured at the surface of the well plate using a PM 160 wireless handheld power meter (Thorlabs, Germany) and was determined to be 4 mW cm^{-2} . This intensity lies within the range commonly used for aPDT and avoids unwanted thermal effects or rapid photobleaching associated with high-intensity light sources.

4.4.2. Efficiency of ROS generation

PhB is widely reported to generate primarily $^1\text{O}_2$ upon photoexcitation.^{174,175} To assess the irradiation time dependence of $^1\text{O}_2$ generation, preliminary experiments were conducted using the chemical probe Singlet Oxygen Sensor Green (SOSG, Scheme 7). SOSG relies on a [4+2] cycloaddition of $^1\text{O}_2$ to its anthracene moiety to

form a fluorescent endoperoxide derivative. Formation of this endoperoxide suppresses intramolecular photoinduced electron transfer (PET), which otherwise quenches the fluorescein fluorophore, resulting in a pronounced increase in fluorescence intensity.



Scheme 7. Interaction of SOSG with $^1\text{O}_2$.

In these experiments, SOSG was incubated with a PhB solution, and the initial fluorescence emission was measured. The sample was then irradiated for defined time intervals using the green LED setup described in 4.4.1, with fluorescence measurements taken after each irradiation step (Figure 11). While SOSG provides a convenient means to compare $^1\text{O}_2$ generation across samples, it is not suitable for absolute $^1\text{O}_2$ quantification. This limitation arises from the intrinsic photosensitivity of SOSG, which can undergo self-conversion to its fluorescent endoperoxide upon green-light irradiation. This process occurs via intramolecular PET followed by ISC, populating the T_1 state of SOSG, which can itself generate $^1\text{O}_2$ through triplet-triplet energy transfer.¹⁷⁶

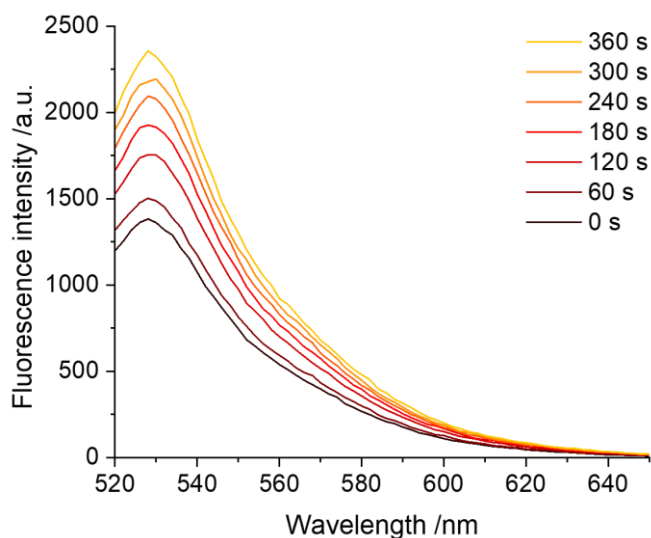


Figure 11. Fluorescence emission spectra ($\lambda_{\text{ex}} = 504 \text{ nm}$) of SOSG ($25 \mu\text{M}$) upon increasing irradiation time with green LEDs ($\lambda = 520 \text{ nm}$, 4 mW cm^{-2}).

Despite this limitation, the $^1\text{O}_2$ generated by PhB under irradiation evidently outweighs the contribution from SOSG self-sensitisation. This is evidenced by a substantial increase in fluorescence intensity relative to the control (Figure 12), which can be attributed to efficient population of the PhB T_1 and subsequent energy transfer to $^3\text{O}_2$. Increasing irradiation time increases SOSG fluorescence intensity, consistent with a time-dependent increase in $^1\text{O}_2$ generation mediated by photoactivated PhB.

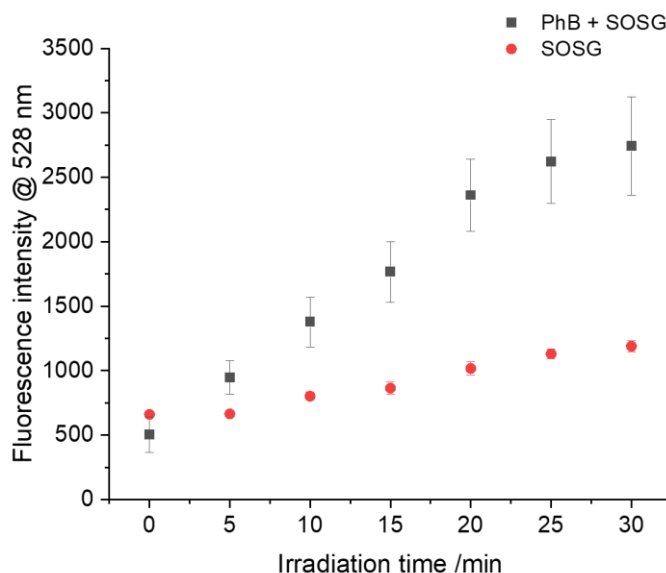


Figure 12. Increase in fluorescence intensity with increasing irradiation time ($\lambda = 520$ nm, 4 mW cm^{-2}) of SOSG ($10 \mu\text{M}$) in the presence and absence of PhB (250 nM).

4.4.3. Effect of irradiation time on aPDT efficacy

Having confirmed that free PhB generates $^1\text{O}_2$ in a time-dependent manner under green light excitation, the next step was to assess whether this photochemically produced $^1\text{O}_2$ translates into effective aPDT activity against model bacterial strains. To address this, the influence of irradiation time and PhB concentration on the antibacterial effect against Gram-positive *S. aureus* was systematically evaluated. The following experiments were performed in simulated thin wound exudate (STWE), an industry-standard medium, also referred to as Solution A, used to mimic wound exudate.^{177,178}

Aqueous PhB solutions were prepared in water and incubated with *S. aureus* RN4220 suspensions ($\sim 10^6 \text{ CFU mL}^{-1}$) in STWE for 15 min to allow dye-cell interaction. Samples were then irradiated for either 9, 90 or 900 s. To mitigate heating effects, longer exposures were delivered in 5-minute intervals (5 min on, 5 min off) (Figure 13). Complete inactivation of *S. aureus* was achieved for irradiation times of 90 s and above across the tested PhB concentration range. This

observation suggests that under these conditions, bacterial killing is limited not by irradiation duration but by the availability of bacterial cells, with shorter irradiation times sufficient for rapid inactivation at lower cell densities.

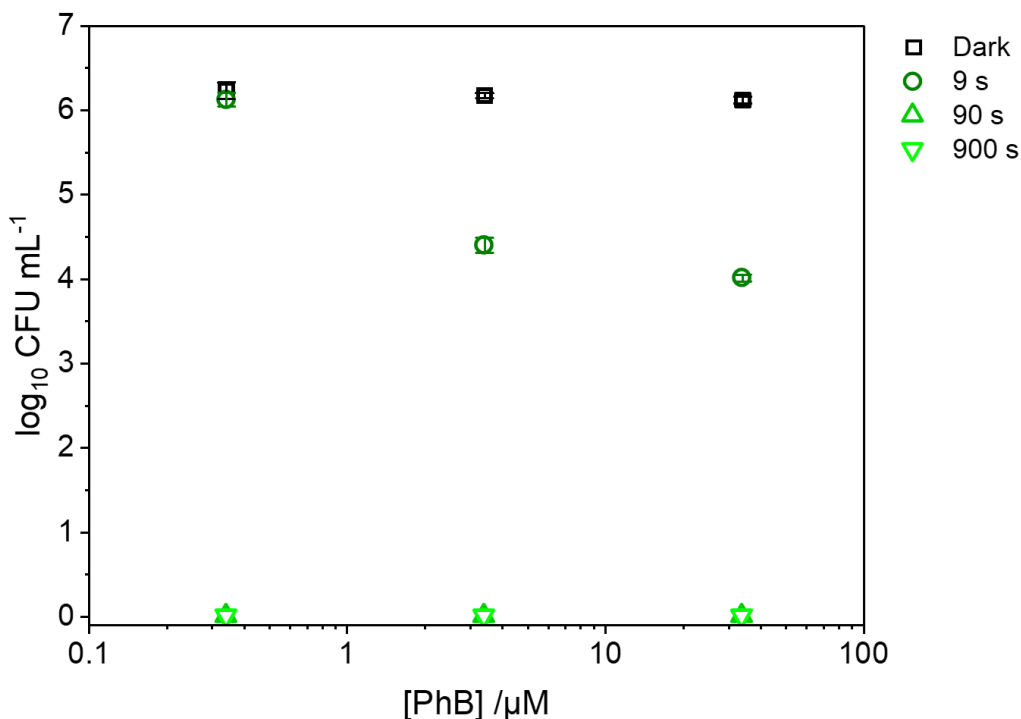


Figure 13. Effect of PhB concentration and irradiation time on *S. aureus* RN4220 viability. CFU counts following aPDT with free PhB at varying concentrations (logarithmic scale) under different irradiation times ($\lambda = 520 \text{ nm}$, 4 mW cm^{-2}). Starting bacterial concentration was $\sim 10^6 \text{ CFU mL}^{-1}$ in STWE. Experiments were performed in technical triplicate.

To further probe the dose-response behaviour, bacterial density was increased to $\sim 10^8 \text{ CFU mL}^{-1}$. At this higher cell density, clear concentration-dependent trends emerged, with CFU counts decreasing as PhB concentration was increased at fixed irradiation times (Figure 14). Notably, a linear decrease in $\log_{10} \text{CFU}$ was observed with increasing PhB concentration at a fixed 90 s irradiation, indicating a power-law relationship between bacterial survival and PhB availability. This represents PS-limited killing, where increased PS molecules or increased irradiation are required to generate sufficient $^1\text{O}_2$ to inactivate the bacteria. These results are consistent with previous reports of PhB-mediated aPDT against *S. aureus*, which also demonstrated strong dependence on PhB concentration and light exposure.¹⁷⁹

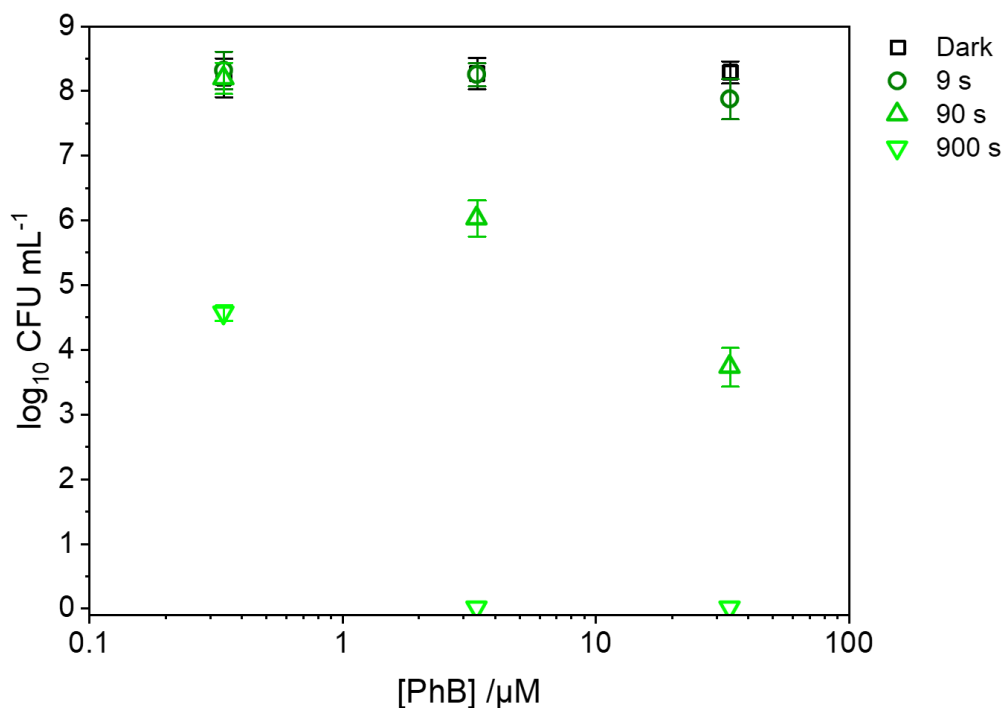


Figure 14. Effect of PhB concentration and irradiation time on *S. aureus* RN4220 viability. CFU counts following aPDT with free PhB at varying concentrations (logarithmic scale) under different irradiation times ($\lambda = 520 \text{ nm}$, 4 mW cm^{-2}). Starting bacterial concentration was $\sim 10^8 \text{ CFU mL}^{-1}$ in STWE. Experiments were performed in technical triplicate.

4.4.4. Efficacy of PhB against other strains of *S. aureus*

To determine whether this antimicrobial activity is conserved across strains with varying antibiotic resistance profiles, the aPDT efficacy was investigated with 2 additional *S. aureus* strains under the same conditions. *S. aureus* eMRSA9 is a multidrug-resistant clinical isolate known to be resistant to multiple antibiotics, including penicillin, tetracycline, erythromycin, gentamycin and kanamycin.¹⁸⁰ *S. aureus* 2569 is a reference strain known to be resistant to colistin and nystatin, frequently used as a benchmark in literature.¹⁸¹

The previous experiment indicated that 15 min irradiation of PhB at a concentration of $3.4 \mu\text{M}$ was sufficient to eradicate *S. aureus* RN4220. As shown in Figure 15, the same conditions completely inactivated both eMRSA9 and 2569, independent of their antibiotic resistance profiles. This demonstrates that photodynamic

inactivation operates via mechanisms distinct from those of conventional antibiotics, relying on oxidative damage rather than on specific metabolic or enzymatic pathways. It has been suggested that xanthene dyes, including PhB, may increase the permeability of *S. aureus* cell membranes to K^+ ions, potentially enhancing oxidative stress and contributing to rapid cell death.¹⁷⁹ Consequently, PhB-mediated aPDT represents a promising antibiotic-independent approach for the treatment of multidrug-resistant pathogens in wound infections.

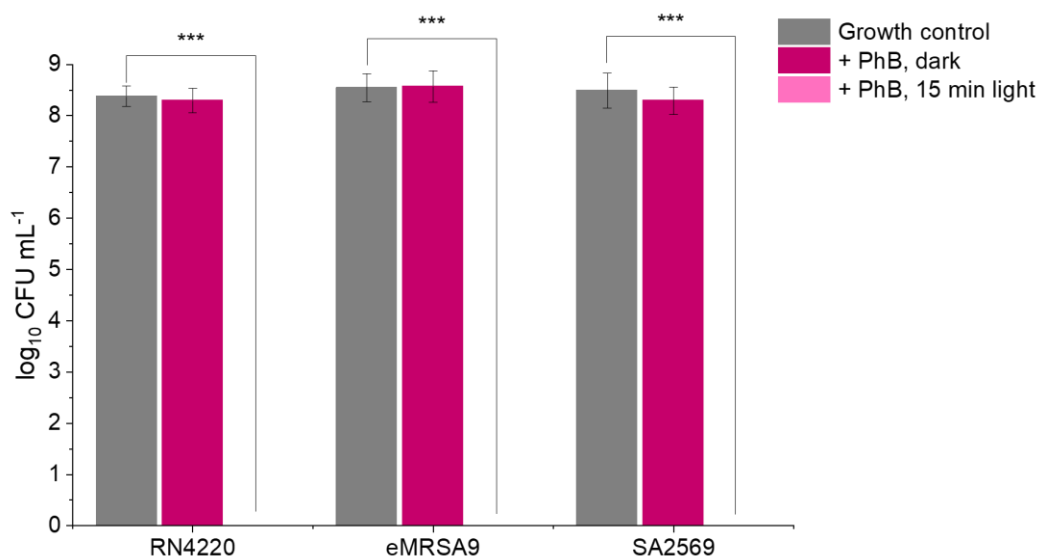


Figure 15. CFU counts of three *S. aureus* strains upon treatment with PhB (3.4 μ M) relative to the growth control without PhB. Samples treated with PhB were either kept in the dark or irradiated for 15 min ($\lambda = 520$ nm, 4 mW cm⁻²). Starting bacterial concentration was $\sim 10^8$ CFU mL⁻¹ in STWE. Asterisks represent significant difference from non-irradiated growth control (***: $p < 0.001$).

4.4.5. Effect of aPDT with PhB against Gram-negative bacteria

While irradiation of PhB has been shown to inactivate multiple *S. aureus* strains, including multidrug-resistant isolates, Gram-positive (monoderm) bacteria are often considered more susceptible to oxidative stress from aPDT due to the absence of an outer membrane. They possess a single lipid membrane, a thick, crosslinked peptidoglycan layer and lipoteichoic acids, which act as chelating agents (Figure 16).^{182,183} In contrast, Gram-negative (diderm) bacteria have both an

inner and an outer cell membrane, a thin peptidoglycan layer, and outer membrane components such as lipopolysaccharides and porins.^{182,184} Accordingly, Gram-negative bacteria are notoriously more difficult to treat with aPDT. Hence, to evaluate the broader efficacy of PhB, its antimicrobial activity was assessed against clinically relevant Gram-negative species.

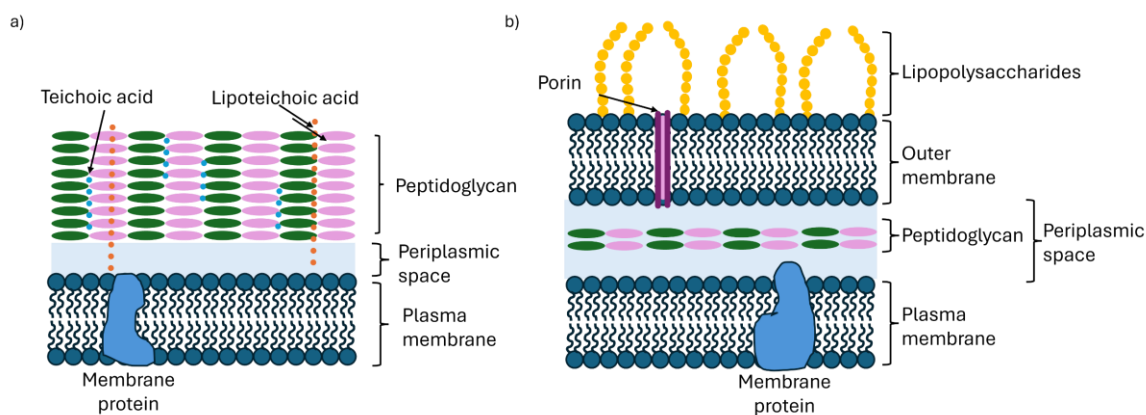


Figure 16. Cell wall structures of a) Gram-positive and b) Gram-negative bacteria.

Pseudomonas aeruginosa

The model strain *P. aeruginosa* ATCC19660 was selected to represent a clinically relevant Gram-negative pathogen associated with wound infections. A low starting inoculum ($\sim 10^6$ CFU mL⁻¹) was incubated with PhB and subjected to either 15 min irradiation or kept in the dark. As shown in Figure 17, 15 min irradiation led to partial inactivation of *P. aeruginosa* at 3.4 μ M, and complete inactivation at 34 μ M. This trend resembles that observed for 9 s irradiation of *S. aureus* (Figure 13), indicating that *P. aeruginosa* is less susceptible and requires longer exposure or higher PhB concentrations to achieve comparable killing. Nevertheless, this data confirms that PhB-mediated aPDT is feasible under adjusted experimental conditions. The eradication of *P. aeruginosa* in these experiments was unexpected, as neat PhB is commonly reported to be active only against Gram-positive bacteria.¹⁶¹ Gram-negative bacteria, including *P. aeruginosa*, are generally more difficult to kill due to

their cell wall structure. A combination treatment of aPDT with chelating agents, antibiotics or antimicrobial peptides to permeabilise the cell wall and facilitate PS uptake has been proposed.^{185–187} The use of neat PhB to photodynamically inactivate *P. aeruginosa* has not yet been reported. In general, Gram-negative bacteria are often resistant to neutral and anionic PSs.¹⁸⁷ Antimicrobial activity of PhB has been observed against Gram-negative bacteria in the presence of EDTA, a chelating agent that binds cations holding the lipopolysaccharides (LPS) of the cell wall in place, thereby increasing cell permeability and enabling dye penetration.¹⁶¹ Possibly the Ca^{2+} ions in STWE in this experiment are permeabilising the LPS, a process known and used for decades in DNA transformation of cells.¹⁸⁸ Moreover, the presence of Cl^- counterions in the STWE may be providing a synergistic effect, as they can combine with the singlet oxygen to produce the longer-lived hypochlorite, ClO^- , which can also have an antimicrobial effect.^{189,190}

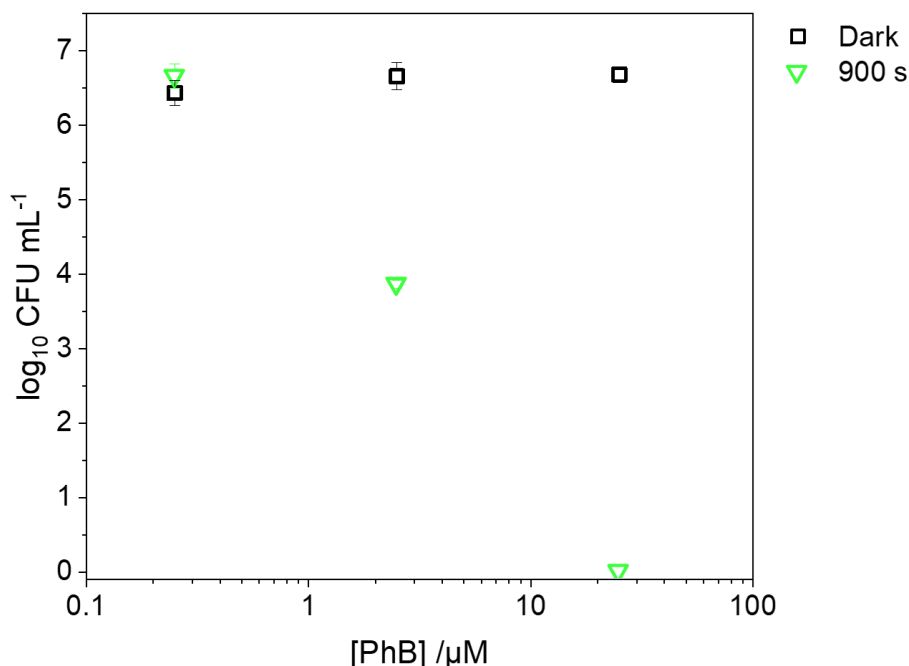


Figure 17. Effect of PhB concentration and irradiation time on *P. aeruginosa* ATCC19660 viability. CFU counts following aPDT with free PhB at varying concentrations (logarithmic scale) under different irradiation times ($\lambda = 520 \text{ nm}$, 4 mW cm^{-2}). Starting bacterial concentration was $\sim 10^6 \text{ CFU mL}^{-1}$ in STWE. Experiments were performed in technical triplicate.

Escherichia coli

To further explore Gram-negative activity, two *E. coli* strains (non-pathogenic MACH1 and pathogenic NCTC10418) were tested under identical experimental conditions. Both strains exhibited complete inactivation upon PhB irradiation effective bacterial killing was also observed following PhB irradiation (Figure 18). These results demonstrate that PhB-mediated aPDT can inactivate multiple bacterial species, including Gram-negative organisms, despite their outer membrane barriers. This highlights the robustness and broad-spectrum potential of PhB as a PS for wound infections where both Gram-positive and -negative pathogens are prevalent.

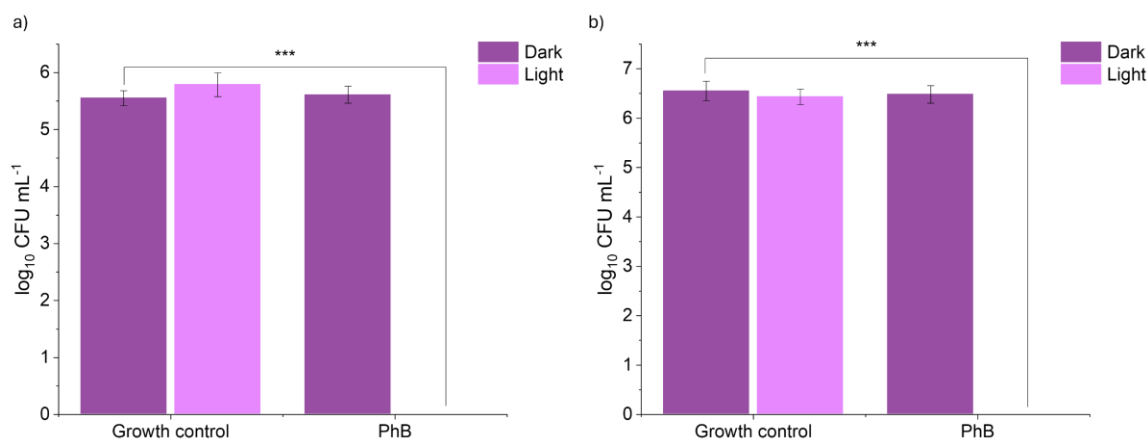


Figure 18. CFU counts of two *E. coli* strains, a) non-pathogenic MACH1 and b) pathogenic NCTC 10418. Samples treated with PhB (34 μ M) were either kept in the dark or irradiated for 15 min (λ = 520 nm, 4 mW cm⁻²). Starting bacterial concentration was $\sim 10^6$ CFU mL⁻¹ in STWE. Asterisks represent significant difference from non-irradiated growth control (***: p < 0.001).

4.4.6. Influence of environmental parameters on aPDT efficacy

The preceding sections demonstrated that the efficacy of PhB-mediated aPDT depends strongly on bacterial species and irradiation dose, with Gram-negative bacteria exhibiting reduced susceptibility relative to *S. aureus*. Beyond intrinsic cellular differences, environmental factors such as oxygen availability and the

physicochemical properties of the surrounding medium are also known to influence the aPDT efficacy and are particularly relevant when considering translation into wound environments. Although molecular oxygen is a fundamental requirement for aPDT, experiments under hypoxic conditions were not conducted in this study, as the oxygen dependence of PhB-induced aPDT is well established in the literature.¹⁷⁹ Instead, the focus was on elucidating trends in aPDT efficacy as a function of PhB concentration, irradiation dose, bacterial load, and medium composition. While the first three were discussed in 4.4.3, a discussion on the influence of the medium is presented here.

Influence of medium composition

Both *S. aureus* and *P. aeruginosa* were initially cultured in LB medium. To assess the impact of medium composition on aPDT efficacy, overnight cultures were centrifuged to obtain bacterial pellets, which were subsequently resuspended in saline, LB or simulated wound fluid (SWF) prior to treatment. In saline, irradiation for 15 min was sufficient to eradicate *S. aureus* across all PhB concentrations tested (Figure 19). In contrast, in LB medium, which is composed of tryptone (30 g L⁻¹), yeast (15 g L⁻¹) and NaCl (30 g L⁻¹), higher PhB concentrations were required to achieve complete killing. However, even sub-micromolar concentrations of PhB produced a marked reduction in viable cell counts, indicating that aPDT activity was retained despite the increased medium complexity. Such medium-dependent performance differences have been previously reported. For example, methylene blue was found to be more efficient against *E. coli* and *Staphylococcus capitis* in PBS than in LB medium, where concentrations as high as 100 µM failed to induce killing after 10 min irradiation with pulsed red LEDs.¹⁹¹ These observations are commonly attributed to competitive light absorption, ROS quenching, and reduced PS-cell interactions in nutrient-rich media.

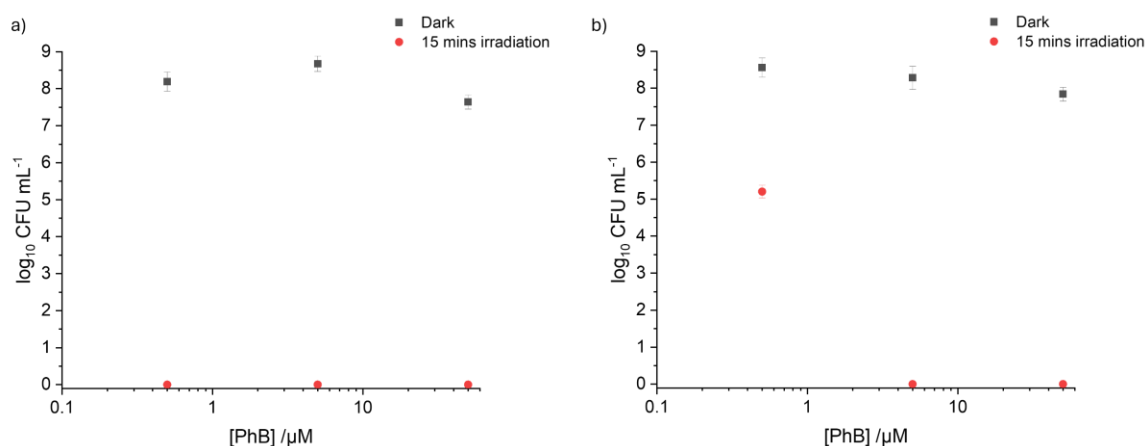


Figure 19. Effect of PhB concentration (logarithmic scale) on *S. aureus* RN4220 viability depending on growth media. Starting bacterial concentration was $\sim 10^8$ CFU mL^{-1} in a) saline and b) LB medium. CFU counts following treatment with free PhB under different irradiation conditions (Dark, or 15 min irradiation ($\lambda = 520$ nm, 4 mW cm^{-2})). Experiments were performed in technical triplicate.

In contrast, *P. aeruginosa* exhibited substantially lower susceptibility to PhB-mediated aPDT. Despite a lower starting inoculum ($\sim 10^6$ CFU mL^{-1}), higher PhB concentrations were required to achieve complete eradication in saline (Figure 20). When the same experiments were performed in LB medium, no measurable loss of viability was observed, indicating insufficient photodynamic damage under these conditions. This behaviour is consistent with previous reports indicating that Gram-negative species are more tolerant to external oxidative stress from aPDT due to their outer membrane enriched in negatively charged lipopolysaccharides, which limits photosensitiser association and restricts diffusion of $^1\text{O}_2$ to cellular components.¹⁹¹ Since effective killing of *S. aureus* was still observed in LB medium, complete quenching of $^1\text{O}_2$ by the medium alone is unlikely to account for this observation. Instead, the reduced efficacy against *P. aeruginosa* implies weaker PhB-cell associations, which could mean the $^1\text{O}_2$ is generated too far from the cellular targets to induce damage. Alternatively, a higher concentration of $^1\text{O}_2$, achieved by increasing PhB concentrations or prolonging irradiation, could help overcome this barrier.

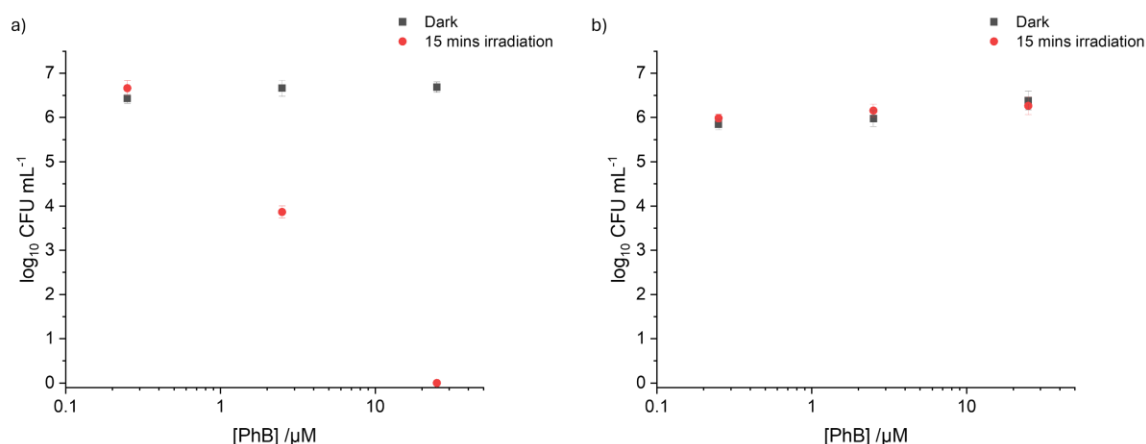


Figure 20. Effect of PhB concentration (logarithmic scale) on *P. aeruginosa* ATCC19660 viability depending on growth media. Starting bacterial concentration was $\sim 10^6$ CFU mL $^{-1}$ in a) saline and b) LB medium. CFU counts following treatment with free PhB under different irradiation conditions (Dark or 15 min irradiation ($\lambda = 520$ nm, 4 mW cm $^{-2}$)). Experiments were performed in technical triplicate.

In SWF, composed of a 1:1 mixture of foetal bovine serum (FBS) and maximum recovery diluent (MRD), neither *S. aureus* nor *P. aeruginosa* was eradicated under any of the conditions tested (Appendix Figure 2). This loss of efficacy is attributed to the complex biological composition of SWF, which can attenuate light penetration, competitively absorb excitation photons, and quench any $^1\text{O}_2$ produced by reaction with proteins and lipids. Similar observations have been reported in protein-rich media such as brain heart infusion broth, which markedly reduced the antibacterial efficacy of deuteroporphyrin compared with a low-protein broth.¹⁰¹ These findings underscore the significant challenges posed by wound environments for aPDT strategies and highlight the importance of PS localisation.

4.4.7. Influence of viscosity and refractive index on aPDT efficacy of PhB

To further elucidate the reduced aPDT activity of PhB against *S. aureus* in LB medium observed in Figure 19, a systematic investigation was conducted in which the antibacterial effect was evaluated in each individual LB component (saline, tryptone and yeast), as well as in all binary and ternary combinations. The resulting data were expressed as log reduction factors (LRFs) to facilitate direct comparison

across conditions to identify any potential trends (Figure 21). With the exception of saline alone, all individual components and their combinations resulted in a diminished aPDT effect relative to that observed in LB medium. This suggests that no single component is responsible for the killing observed in LB.

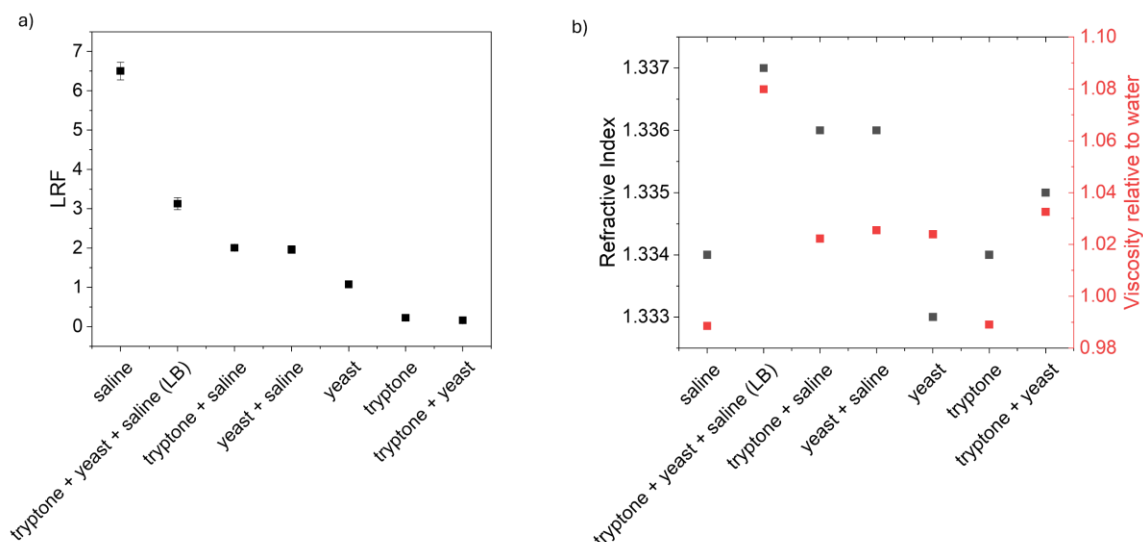


Figure 21. Investigation of the individual components of LB medium. a) Log reduction factors obtained upon incubation of *S. aureus* RN4220 with aqueous PhB (500 nM) in the corresponding media. b) The measured refractive indices and viscosities of each component and combination.

To assess whether bulk optical or rheological properties of the medium could account for these observations, two physicochemical parameters, refractive index (RI) and viscosity relative to water, were determined for each solvent and compared to the corresponding LRF values. However, no clear correlation was observed. Notably, LB exhibited the highest RI and viscosity among the tested media, whereas saline showed both the lowest viscosity and one of the lowest RIs. These findings indicate that neither RI nor viscosity alone directly governs the antibacterial activity. Instead, the results suggest that more complex medium-dependent effects, such as oxygen availability or competitive quenching of $^1\text{O}_2$, are likely to dominate the observed activity.

4.5. Interaction of PhB with *S. aureus* vs *P. aeruginosa*

Previous studies have demonstrated that the potency of aPDT correlates with the extent of PS association with bacterial cells, as $^1\text{O}_2$ has a short lifetime and diffusion length in biological media.¹⁷⁹ To determine whether the observed differences in PhB-mediated aPDT efficacy between *S. aureus* and *P. aeruginosa* arise from differential interactions with PhB, a fluorescence-based partitioning experiment was performed.

Overnight cultures of *S. aureus* and *P. aeruginosa* were resuspended in saline and adjusted to an OD₆₀₀ corresponding to $\sim 10^8$ CFU mL⁻¹. The bacterial suspensions were then incubated with an equal volume of PhB solution (5 μM) for either 15 min or 16 h at 4 °C. As a control, the same PhB solution was incubated with an equal volume of saline in the absence of bacteria. Following incubation, the samples were centrifuged to pellet the bacteria. The supernatants were collected and their fluorescence emission spectra measured to assess the fraction of unbound PhB. The bacterial pellets were subsequently resuspended in the original volume of saline, and the fluorescence emission of the resuspended pellets was measured (Figure 22).

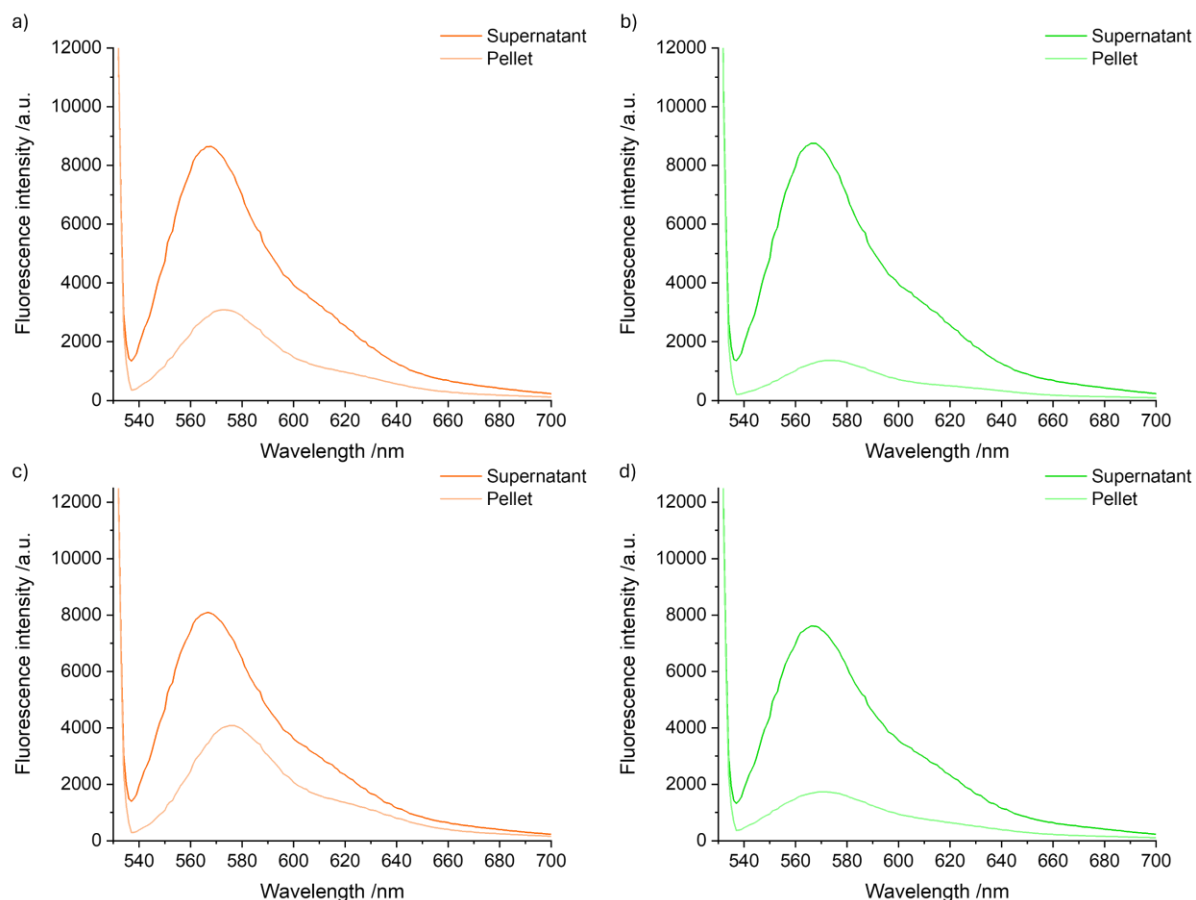


Figure 22. Assessment of PhB association with bacterial cells by fluorescence partitioning. a, c) *S. aureus* and b, d) *P. aeruginosa* suspensions ($\sim 10^8$ CFU mL $^{-1}$) were incubated with PhB (5 μ M) for a-b) 15 min or c-d) 16 h, followed by centrifugation to separate bacterial pellets and supernatants. Fluorescence emission spectra were recorded for the supernatants and for resuspended bacterial pellets ($\lambda_{\text{ex}} = 504$ nm).

Control experiments without bacteria indicated that some PhB was not recovered from the Eppendorf tubes when collecting the supernatant (Appendix Figure 3). This minor loss, accounting for <8% of the total fluorescence, is attributed to adsorption of PhB onto the walls of the tube and establishes the measurement's baseline uncertainty. Importantly, bacterial suspensions in the absence of PhB exhibited negligible fluorescence emission at these wavelengths (intensity < 150 a.u., Appendix Figure 4), confirming that the fluorescence detected in these experiments originates from PhB rather than intrinsic bacterial autofluorescence.

A clear difference in PhB association was observed between the two bacterial species. *S. aureus* pellets exhibited substantially higher fluorescence intensities than those of *P. aeruginosa*, indicating a greater interaction between PhB and the Gram-positive cells. In contrast, the fluorescence emission intensities measured from *P. aeruginosa* pellets were comparable to those observed in the control experiments without bacteria, suggesting that the detected signal may largely arise from non-specific adsorption, rather than from cellular association.

To enable direct comparison, pellet-associated fluorescence intensities were normalised to the total fluorescence (pellet + supernatant) for each condition (Figure 23). For *S. aureus*, more than 25% of the total fluorescence emission was associated with the pellet, more than double the fraction observed for *P. aeruginosa*. This indicates a markedly greater interaction between PhB and the Gram-positive bacterium and can be attributed to differences in cell envelope architecture. It is also consistent with the higher susceptibility of *S. aureus* to PhB-mediated aPDT observed in Sections 4.4.3 to 4.4.7.

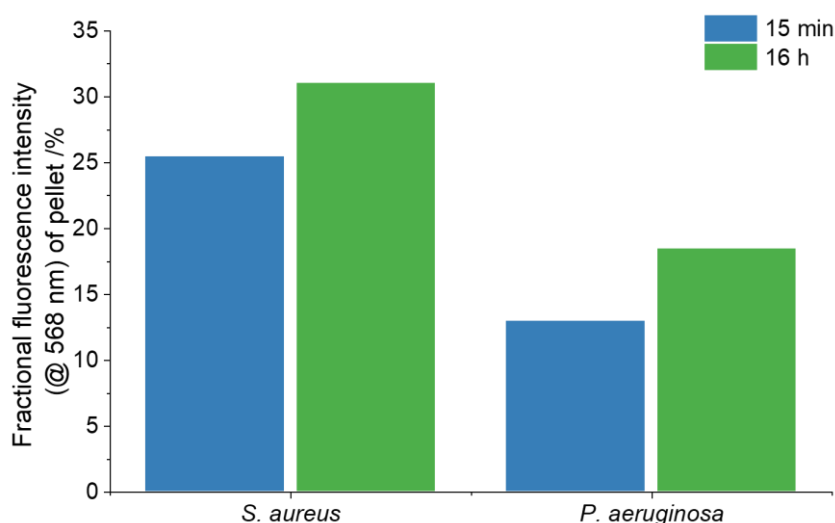


Figure 23. Fraction of total fluorescence associated with the bacterial pellet following incubation with PhB (15 μ M) for 15 min or 16 h.

The influence of incubation time was also evaluated. Extending the incubation period from 15 min to 16 h resulted in only a modest increase (~6%) in the pellet-

associated fluorescence for both species, despite a 64× increase in incubation duration. This minimal change indicates that PhB association with bacterial cells occurs rapidly and does not increase substantially with prolonged exposure. This could also indicate that the interaction involves surface association rather than active uptake into the bacterial cell. Overall, these results indicate that PhB exhibits a limited association with *P. aeruginosa* under the conditions studied, while interactions with *S. aureus* are more pronounced.

4.6. Summary and conclusion

In this Chapter, the photophysical properties, photostability, and aPDT activity of free PhB were systematically characterised to establish baseline benchmarks for subsequent polymer-based photodynamic systems. PhB exhibits strong absorption in the green region of the visible spectrum, enabling efficient activation with low-cost LED arrays, confirming its suitability as a $^1\text{O}_2$ -generating PS.

Baseline aPDT experiments demonstrated that free PhB can induce effective bacterial killing under optimised conditions. However, its antimicrobial efficacy was strongly dependent on concentration, light dose, bacterial load and environmental parameters. In particular, reduced activity was observed in nutrient-rich and wound-mimicking media, as well as against Gram-negative bacteria, highlighting the sensitivity of free PhB-based aPDT to diffusion, dilution and the lack of localisation to bacterial cells.

These findings collectively demonstrate that while free PhB is an effective PS, its poor retention and localisation constrain its practical application. This motivates the development of strategies that enhance PhB proximity to bacterial targets while retaining photodynamic activity. The constraints identified in this Chapter directly motivate the use of polymeric vesicles and surface-immobilised systems explored in the following Chapters, in which controlled localisation and retention of PhB are leveraged to overcome the intrinsic limitations of free PS.

4.7. Materials and methods

4.7.1. Materials

Phloxine B (2',4',5',7'-tetrabromo-4,5,6,7-tetrachlorofluorescein disodium salt, dye content $\geq 80\%$), rhodamine 6G (9-[2-(ethoxycarbonyl)phenyl]-*N*-ethyl-6-(ethylamino)-2,7-dimethyl-3*H*-xanthen-3-iminium chloride, dye content $\sim 95\%$), phosphoric acid (H_3PO_4 , 99%) boric acid (H_3BO_3 , ACS reagent, $\geq 99.5\%$), sodium acetate (NaOAc , $\geq 99\%$) and dimethyl sulfoxide (DMSO, 99%) were purchased from Sigma-Aldrich (Merck, Germany). Sodium hydroxide (NaOH , $>98.8\%$) and hydrochloric acid (HCl) were purchased from Carl Roth, Germany. Methanol ($>99.8\%$) was purchased from VWR, Germany. Singlet Oxygen Sensor Green SOSG was purchased from Thermo Fisher Scientific, USA). Tris buffer (100 mM, pH 7.4) was purchased from Millipore, USA.

Lysogeny broth (LB, Luria/Miller: 10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 10 g L^{-1} NaCl, pH 7.0 ± 0.2) and LB agar (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 10 g L^{-1} NaCl, 15 g L^{-1} agar-agar, pH 7.0 ± 0.2) were purchased from Carl Roth, Germany. Sterile water and 0.9% saline were purchased from Versylene Fresenius, Germany.

Acrylic and polystyrene cuvettes and transparent flat-bottom 96-well plates were purchased from Sarstedt, Germany, and cell culture 96 black half-area μ -clear well plates from Greiner Bio-One, Germany.

Milli-Q water was drawn from a Millipore Direct Q8 system with a resistivity of $\geq 18.2 \text{ M}\Omega \text{ cm}$ (Millipore Advantage A10 system, Schwalbach, with Millimark Express 40 filter, Merck, Germany).

4.7.2. Bacterial strains

S. aureus lab strain RN4220 (ATCC 35556) was originally derived from NCTC 8325-4 via UV and chemical mutagenesis and was purchased from Leibniz-Institute DSMZ GmbH (Germany). *S. aureus* clinical isolate eMRSA 9 was isolated from an invasive

S. aureus outbreak in John Radcliffe Hospital in Oxfordshire, UK, collected between 1997-1998. It has known resistances to penicillin, tetracycline, erythromycin, kanamycin and gentamycin. *S. aureus* reference strain 2569 (ATCC 29213, strain designation: Wichita) is an MSSA strain isolated from an infected wound, purchased from Leibniz-Institute DSMZ GmbH (Germany).

Pseudomonas aeruginosa lab strain ATCC 19660 (isolated from sepsis in Lima, Peru) was purchased from LGC Standard GmbH (Germany).

The pathogenic *E. coli* clinical isolate NCTC 10418 (ATCC 10536, strain designation: MacLeod) was purchased from Leibniz-Institute DSMZ GmbH (Germany). The non-pathogenic *E. coli* MACH1™ (Invitrogen) was purchased from Thermo Fisher Scientific (USA).

4.7.3. UV-Vis spectroscopy

Absorbance measurements were performed on a Varian Cary 50 Bio spectrometer (Varian Inc., USA). Spectra were recorded in 1 cm path length polystyrene cuvettes at 25 °C with a scan rate of 300 nm min⁻¹.

4.7.4. Fluorescence spectroscopy

Emission spectra were recorded using a Varian Cary Eclipse spectrometer (Varian Inc., USA) in acrylic cuvettes at 25 °C with a scan rate of 120 nm min⁻¹.

4.7.5. Determination of fluorescence quantum yield

Aqueous solutions of R6G and PhB were adjusted to be of equal absorbance at 504 nm. These solutions were then excited at 504 nm, and the emission was measured from 510 nm – 700 nm. Using Origin, the emission spectra were integrated to obtain the area, which was used to calculate the quantum yield of PhB relative to R6G using Equation 3.

4.7.6. Investigation of pH-dependent behaviour of PhB

Britton Robinson (BR) buffer was prepared by dissolving H_3PO_4 , H_3BO_3 , and NaOAc (0.04 M each) in Milli-Q water. A bulk solution of PhB (20 μM , 200 mL) was prepared in BR buffer, then adjusted to pH 1.1 with HCl (2.4 M). The initial absorbance of PhB at pH 1.1 was recorded using a 1 cm quartz cuvette. The contents of the cuvette were returned to the bulk solution, which was titrated with small aliquots of NaOH (6.1 M) to increase the pH gradually ($0.1 < \Delta\text{pH} < 0.5$). Absorption measurements were repeated after each titration, ensuring thorough rinsing of the cuvette with the measuring solution before measuring.

4.7.7. Photostability of PhB

A solution of PhB was prepared in PBS, and its concentration was adjusted to an extinction of ~ 1 in a sealed 1 cm quartz cuvette. The cuvette was placed on an overhead projector (Liesegang Trainer Special HR Electronic Overhead Projector) fitted with a 575 W mercury halide lamp (Appendix Figure 1). Absorption spectra were measured every 30 minutes for 4 hours, then again after 24 hours. The ratio of the absorption at $\lambda_{\text{max}} = 539 \text{ nm}$ at each time point (A_0) to the initial absorption (A_t) was used to fit the data to a pseudo-first order kinetic plot, using Equation 4, yielding an apparent first-order rate constant, k .

$$\ln\left(\frac{A_0}{A_t}\right) = kt \quad \text{Equation 4}$$

To determine the half-life ($t_{1/2}$) of PhB under these conditions, k was converted into $t_{1/2}$ using Equation 5.

$$t_{1/2} = \frac{\ln(2)}{k} \quad \text{Equation 5}$$

4.7.8. SOSG assay

To detect $^1\text{O}_2$ production, the probe SOSG was used. PhB was mixed in a 9:1 v/v ratio with SOSG (100 μM in Tris) to afford a final SOSG concentration of 10 μM in a black half-area μ -clear 96-well plate. Fluorescence intensity was measured from the top using a Tecan SAFIRE microplate reader (Tecan, Switzerland) with an excitation of 504 nm and emission was measured from 520 – 645 nm to collect spectra or at 528 nm for point measurements. The samples were irradiated in 5-minute intervals, with fluorescence measurements performed between each irradiation.

4.7.9. Antibacterial activity

Overnight bacterial cultures were prepared by inoculating LB (5 mL) with a single bacterial colony and incubating for 18 h at 37 °C while shaking at 200 rpm. To exchange the media, overnight cultures were centrifuged (10000 rpm, 5 min), the supernatant discarded and the pellet resuspended in the required media. The OD_{600} of the bacterial suspensions was adjusted to 1.2 (*S. aureus*) or 0.6 (*P. aeruginosa*, *E. coli*) by dilution in the working medium, which corresponds to $\sim 10^8$ CFU mL^{-1} . Working concentrations were obtained by further dilutions in the working medium (e.g. 100 $\times$ dilution for a 10^6 CFU mL^{-1} suspension). To evaluate the aPDT efficacy of PhB, 25 μL PhB was incubated with 25 μL bacteria suspension for 15 min at 21 ± 1 °C. Irradiation was performed by placing the light setup directly on top of the well plate without a lid. All experiments were performed in technical triplicate, with separate plates for light and dark conditions. To count remaining bacteria, 10 μL from each well, and 10-fold serial dilutions of each well, were spotted 3 $\times$ onto LB agar plates, resulting in a limit of detection of 34 CFU mL^{-1} . After incubation (37 °C, overnight) of these LB agar plates, colonies were counted and the $\log_{10}$ CFU mL^{-1} for each well was calculated. Biological triplicates (3 $\times$ independently performed experiments) afforded the mean and standard deviation.

4.7.10. Determination of refractive index

The refractive index (RI) of saline, individual LB medium components (tryptone and yeast) and their combinations was measured using an Abbe Refractometer (Carl Zeiss, Germany). The refractometer was calibrated at room temperature (20 – 22 °C) using Milli-Q water (RI = 1.3330 at 20 °C) according to the manufacturer's instructions. For each measurement, a small aliquot (2-3 drops) of the test medium was placed onto the cleaned prism surface, ensuring complete coverage without air bubbles. The prism assembly was closed, and the RI was read directly from the instrument scale. All measurements were performed under identical ambient conditions to minimise temperature-dependent variations. Each sample was measured in triplicate, with the prism cleaned (Milli-Q water, EtOH) and dried between measurements.

4.7.11. Partitioning of PhB in bacterial pellets

To investigate the interaction of PhB with Gram-negative vs Gram-positive bacteria, suspensions of *P. aeruginosa* and *S. aureus* were prepared in saline as described in Section 4.7.9. A PhB solution (5 µM in Milli-Q) was incubated in equal volumes (0.75 mL each) with a bacterial suspension in a 2 mL polypropylene Eppendorf tube. PhB with saline was used as a control. This mixture was vortexed for 30 s, then left at 4 °C for either 15 min or 16 h. After the incubation period, the Eppendorf tubes were centrifuged (10000 rpm, 5 min). The supernatant was transferred to a second tube, while the pellet was resuspended in saline (1.5 mL). Fluorescence emission spectra of the supernatants and pellets were measured in a 96-well plate using a Tecan Infinite 200 (λ_{ex} = 504 nm).

5. PhB-loaded PEG-*block*-polyester vesicles for light-triggered aPDT

This Chapter describes the synthesis and characterisation of amphiphilic block copolymers composed of poly(ethylene glycol) (PEG) as the hydrophilic block and poly(lactic acid) (PLA) or poly(ϵ -caprolactone) (PCL) as the hydrophobic polyester block. These biodegradable PEG-*block*-polyester systems were selected to facilitate the self-assembly of micellar and vesicular nanostructures capable of encapsulating cargo, including the PS PhB. The self-assembled PhB-loaded vesicle systems were subsequently evaluated for their aPDT efficacy against *S. aureus* and *P. aeruginosa*.

Parts of this Chapter have been published in RSC Advances.ⁱ

5.1. Objectives

Amphiphilic block copolymers (ABCPs) consist of chemically distinct hydrophilic and hydrophobic segments, which enable spontaneous self-assembly into nanoscale aggregates when dispersed in selective solvents. The formation of thermodynamically stable ABCP assemblies is governed by several parameters, including the degree of stretching of the core-forming blocks, the interfacial tension between the hydrophobic core and the surrounding solvent, and the repulsive interactions between the corona-forming chains. In practice, the morphology of the assemblies formed can be controlled by tailoring the copolymer composition and concentration in solution, as well as the nature of the common solvent and the ratio

ⁱ Cusick, N; Schönherr, H. Phloxine B-loaded polymersomes enable eradication of *Pseudomonas aeruginosa* and *Staphylococcus aureus* in antimicrobial photodynamic therapy. *RSC Advances* **2025**, 15, 18815-18825.

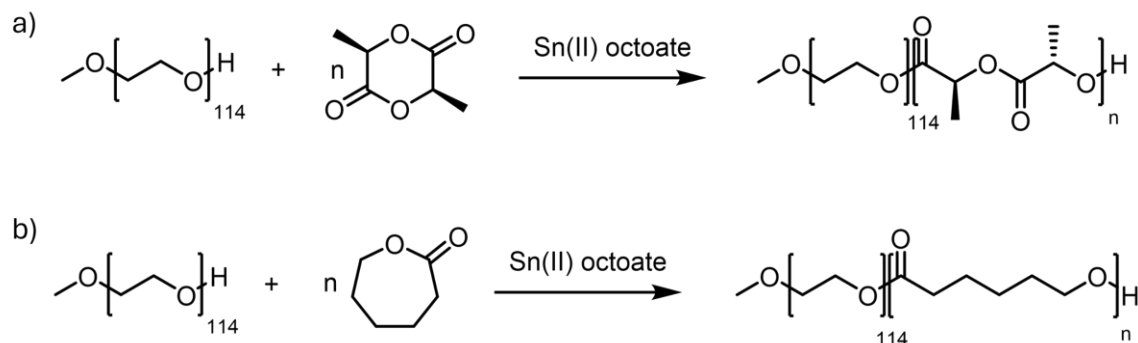
Author contributions: NC: methodology, investigation, formal analysis, validation, visualisation, data curation, writing – original draft; HS: conceptualisation, supervision, funding acquisition, project administration, resources, writing – review & editing.

of common- to anti-solvent. Among these factors, the copolymer composition is the only parameter that must be defined prior to the self-assembly process and therefore represents a key design variable.

In this Thesis, PEG-based block polymers were investigated as model ABCP systems. PEGylation is well known to confer colloidal stability and hydration, while minimising nonspecific bacterial and protein adsorption.¹⁹² PEG is also considered the gold-standard hydrophilic block for biomedical systems, providing a standard that allows changes in assembly behaviour to be attributed to the variations in the hydrophobic block.¹⁴¹ PLA and PCL were selected as the hydrophobic tails due to their biodegradability via ester hydrolysis, biocompatibility, synthetic accessibility and extensive prior use in drug delivery systems.^{193,194} Although both are FDA-approved aliphatic polyesters and can be synthesised and self-assembled using similar methodologies, they differ in key physicochemical properties; PLA exhibits a slightly higher glass transition temperature (T_g) of ~ 330 K vs ~ 213 K, lower crystallinity, and faster hydrolytic degradation rate compared to PCL.^{194–196}

5.2. Synthesis of PEG-*b*-PLA and PEG-*b*-PCL

Cyclic esters, or lactones, are commonly polymerised via ring-opening polymerisation (ROP), in which an alcohol, acid or base initiator opens the strained ester ring to generate a reactive chain end capable of successive monomer addition.¹⁴³ This process is driven by the release of ring strain and is widely employed for the synthesis of the biodegradable, FDA-approved polymers PLA and PCL. In this work, mono methoxy-terminated PEG (PEG methyl ether, mPEG) was used as a macroinitiator, enabling the synthesis of PEG-*b*-PLA and PEG-*b*-PCL BCPs via coordinative ROP (Scheme 8).



Scheme 8. Synthesis of a) PEG-*b*-PLA and b) PEG-*b*-PCL via ROP.

Due to the hydrophilic nature of PEG and the hydrophobic character of PLA and PCL, the resulting BCPs are amphiphilic. A key feature of such ABCPs is their ability to undergo self-assembly in response to changes in solvent conditions.¹⁴¹ Early work by Eisenberg in the 1990s demonstrated that the self-assembly behaviour of poly(styrene)-*block*-poly(acrylic acid) depended on the volume fraction of the hydrophilic and hydrophobic blocks.¹⁹⁷ By tuning this volume fraction, different morphologies, including micelles and vesicles, can be obtained.¹³⁰ In practice, control over the volume fraction is most readily achieved by adjusting the relative chain lengths, and thus the weight ratio, of the two blocks.¹³⁰

Variation in PEG-*b*-PLA and PEG-*b*-PCL BCP composition was therefore achieved by adjusting the monomer:macroinitiator ratio for PLA or PCL, targeting hydrophobic block lengths ranging from 50 to 500 constitutional repeat units. All BCPs were synthesised via coordinative ROP, a well-established and highly controllable method for preparing biodegradable aliphatic polyesters. In each reaction, mPEG acted as a macroinitiator, forming the hydrophilic block, while the hydrophobic polyester block was grown from the terminal hydroxyl group of mPEG. Tin(II) (2-ethylhexanoate)₂ (Sn(Oct)₂), a widely used and FDA-accepted catalyst for medical-grade polyesters, was employed to achieve high monomer conversion.¹⁴³

5.2.1. Synthesis of PEG-*b*-PLA

PEG-*b*-PLA ABCPs were obtained in good yields between 72-91%. Successful synthesis was confirmed with ^1H -NMR, GPC and TGA. An exemplary ^1H -NMR spectrum of PEG-*b*-PLA with a nominal PLA chain length of 500 repeat units is shown in Figure 24. The peaks are well resolved and can be integrated to determine the block lengths. PEG protons are observed as a strong singlet at ~ 3.6 ppm in CDCl_3 (Figure 24). As a commercially available PEG macroinitiator ($M_n = 5000 \text{ g mol}^{-1}$) was used, the integral of this singlet at $\delta = 3.6$ ppm (Figure 24b, Appendix Figure 5) can be normalised to the corresponding number of protons, 456 (114 repeat units $\times$ 4 protons). This enables calculation of the obtained PLA block lengths by the ratio of the integrals of PEG to PLA protons, which present as a quartet at $\delta = 5.2$ ppm, corresponding to the backbone methine protons (Figure 24a) and a doublet at $\delta = 1.6$ ppm corresponding to the methyl protons (Figure 24c).^{198–200} The ^1H -NMR spectrum of L-lactide can be seen in Appendix Figure 6.

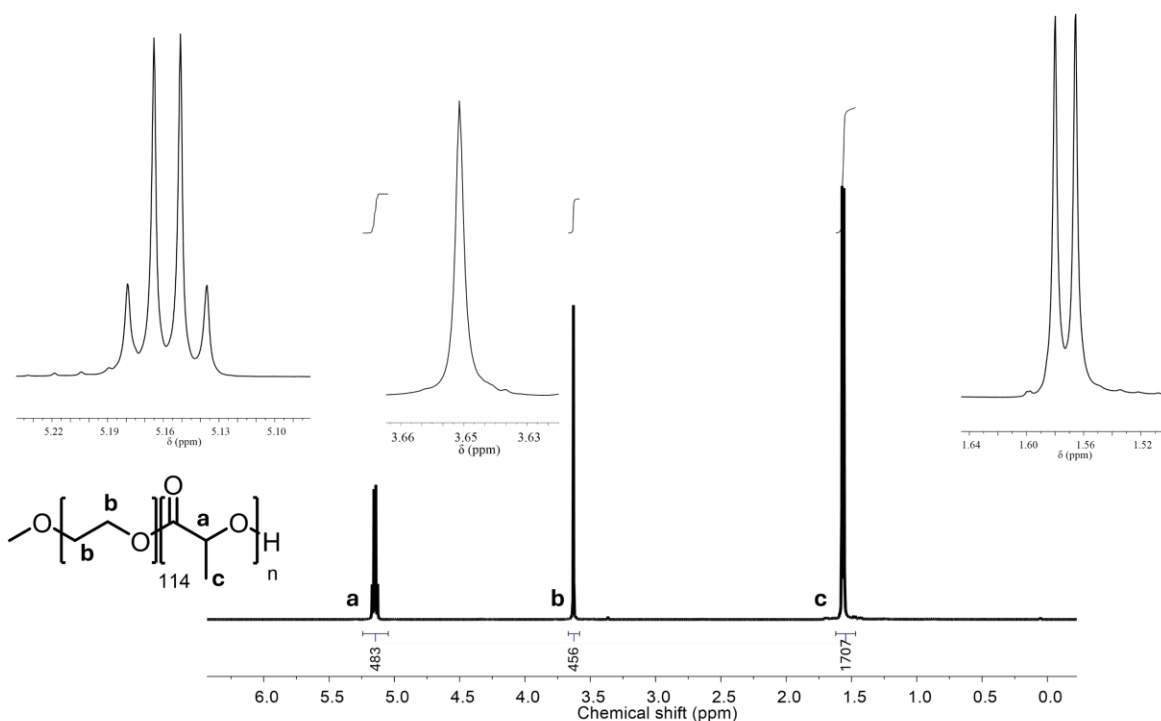


Figure 24. ^1H -NMR Spectrum of PEG₁₁₄-*b*-PLA₅₀₀ in CDCl_3 .

By using this reaction set-up, adjustable PLA block lengths were obtained by varying the ratio of L-lactide:mPEG as shown in Appendix Figure 7. The experimentally obtained PLA block lengths were calculated as 72-98% of the targeted values per **Equation 7** and presented in Table 1. This highlights the fine control over the polymerisation.

Table 1. Number average molecular weight (M_n) and corresponding hydrophobic block length of the synthesised PEG-*b*-PLA polymers according to ^1H -NMR.

Targeted PLA chain length, n	M_n /kDa		PLA block length /number of repeat units	
	Expected	Observed	Expected	Observed
50	8.6	7.6	50	36
100	12.2	11.6	100	92
150	15.8	15.3	150	143
300	26.6	26.2	300	294
400	33.8	32.9	400	389
500	41.0	40.1	500	488

Further confirmation of successful synthesis was provided by ATR-FTIR. By stacking the spectra of the mPEG macroinitiator, a commercially available PLLA homopolymer and the synthesised PEG-*b*-PLA₁₅₀ copolymer, characteristic bands for each block were identified (Figure 25, Appendix Table 1). Importantly, strong C=O stretching bands were observed at $\sim 1749\text{ cm}^{-1}$ for PEG-*b*-PLA₁₅₀, indicative of the formation of the polyesters.²⁰¹ This band is also prominent in PLLA. The characteristic C-O-C ether stretching bands of the PEG block were present at $\sim 1100\text{ cm}^{-1}$ and were also conserved in PEG-*b*-PLA₁₅₀, but overlap with the C-O-C ester stretching from PLA.²⁰² This data therefore confirms that both blocks are present in the synthesised polymers, but cannot differentiate between covalently linked blocks and blends.

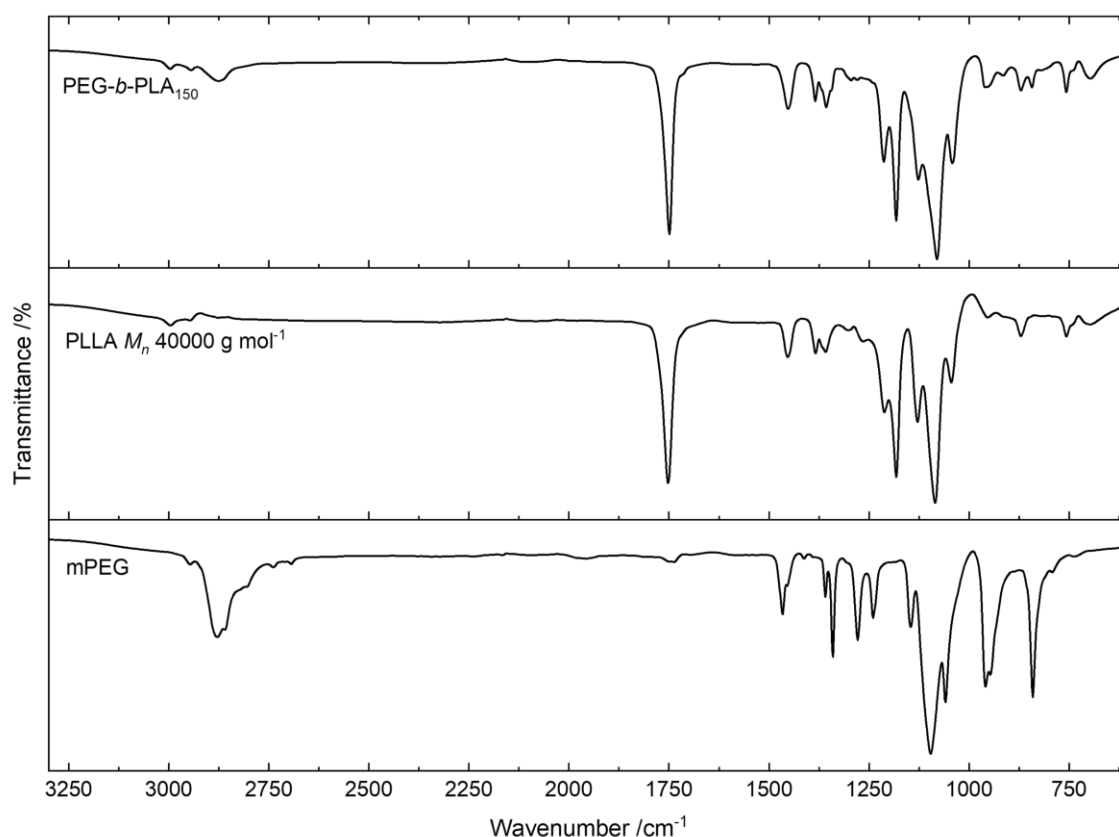


Figure 25. ATR-FTIR spectra of the mPEG macroinitiator, PLLA with a M_n of 40000 g mol⁻¹ (purchased from Sigma-Aldrich, Germany) and the synthesised PEG-*b*-PLA₁₅₀.

GPC was therefore performed in THF for the PEG-*b*-PLA samples with polystyrene standards to confirm controlled block copolymerisation behaviour and assess polydispersity. The mPEG macroinitiator, as well as a PLA homopolymer purchased with an approximate molecular weight of 6 kDa were used to afford subsequent calculation of the obtained PLA block lengths.

The presence of one broad peak in the GPC chromatograms confirmed successful synthesis of the PEG-*b*-PLA copolymers (Figure 26). While the measured molecular weight appears to increase with increasing block length, the calculated PLA block length is consistently significantly greater than expected from ¹H-NMR (Table 2). However, molecular weights determined by GPC should be regarded only as apparent values, as GPC separates polymers based on hydrodynamic volume rather than absolute molecular weight.²⁰³ While the hydrodynamic radius is

proportional to the molecular weight, it is also proportional to the chain conformation.²⁰⁴ ABCPs adopt non-ideal, solvent-dependent conformations resulting in hydrodynamic volumes which are different to the polystyrene standards used for calibration.²⁰⁵ The GPC traces for the synthesised copolymers display non-Gaussian distributions that are typical for block copolymers.²⁰⁶

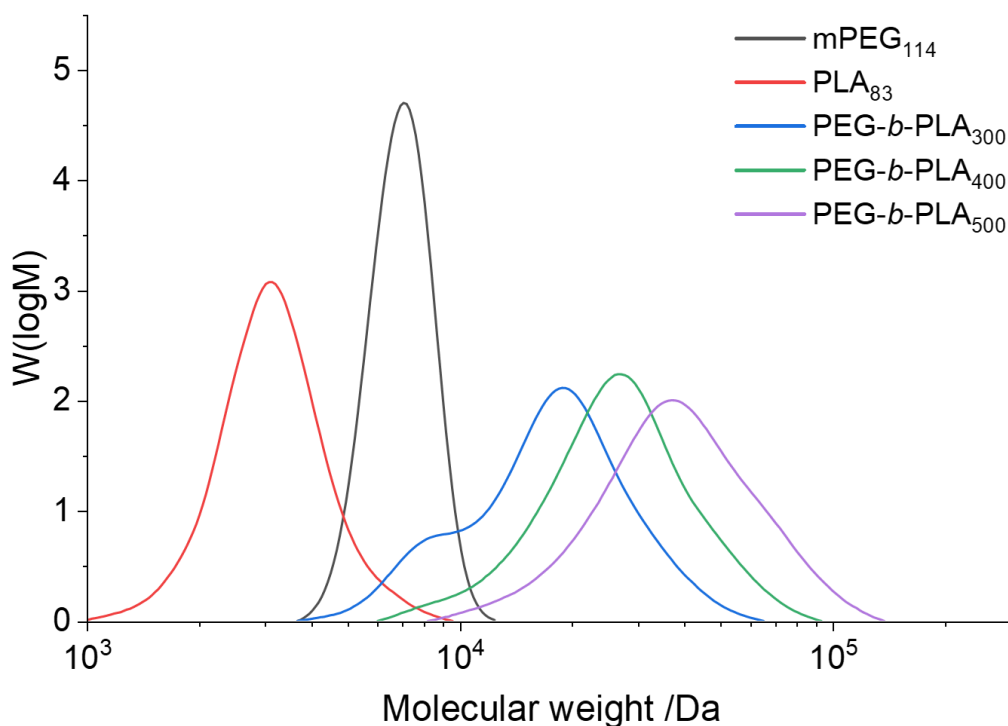


Figure 26. GPC chromatogram of synthesised PEG-*b*-PLA copolymers relative to the mPEG macroinitiator. Measurements were performed in THF with a polystyrene standard.

Table 2. Parameters calculated from the GPC chromatograms of the measured PEG-*b*-PLA copolymers.

	mPEG ₁₁₄	PLA ₈₃	PEG- <i>b</i> -PLA _x		
			x=150	x=300	x=500
<i>Mn</i> /g mol⁻¹	6801	2956	15010	23470	33600
<i>Mw</i> /g mol⁻¹	7048	3296	19120	28830	42220
D	1.04	1.12	1.27	1.23	1.28
Calculated PLA block lengthⁱ	-	-	231	468	752

i: calculated according to **Equation 9**.

In addition to molecular weight and dispersity, the intrinsic thermal stability of the block copolymers was also evaluated. TGA was performed on a representative PEG-*b*-PLA sample. The trace reflects the sequential thermal degradation of the individual blocks (Figure 27).⁵ PEG-*b*-PLA exhibits a sharp degradation step ~ 250 °C, characteristic of PLA degradation, which proceeds via ester pyrolysis, backbiting reactions, and formation of lactide and oligomers.⁶ A more gradual mass loss at ~ 370 - 400 °C corresponds to the degradation of PEG, which occurs via ether bond cleavage and random chain scission.²⁰² The broader, more gradual profile suggests a slower and more distributed degradation process. TGA can also be used to independently assess the mass fraction of the hydrophobic block, providing cross-validation of the copolymer compositions.²⁰⁷ Comparison of the weight fractions results in an estimated 490 PLA repeat units for the PEG-*b*-PLA₅₀₀ polymer, which is in good agreement with the 488 repeat units calculated from $^1\text{H-NMR}$.

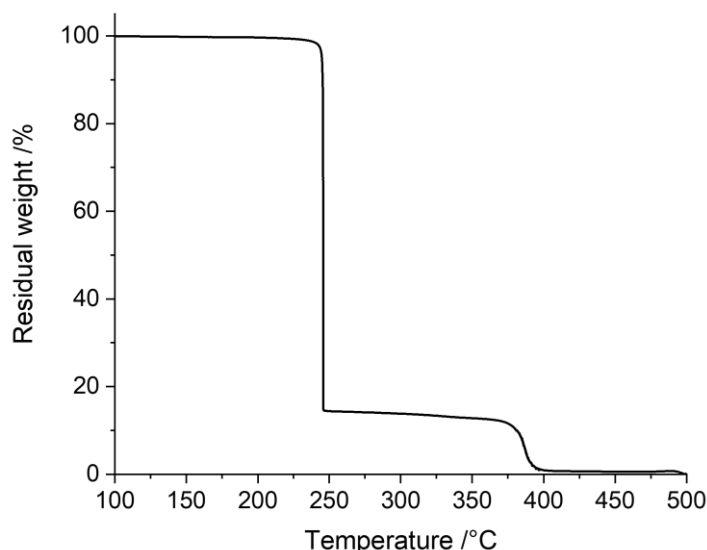


Figure 27. TGA trace of the synthesised PEG-*b*-PLA₅₀₀ block copolymer under N₂ atmosphere (50 °C min⁻¹).

5.2.2. Synthesis of PEG-*b*-PCL

Successful synthesis of PEG₁₁₄-*b*-PCL_x copolymers with varying PCL chain length was also carried out with yields of 76-92%. A typical $^1\text{H-NMR}$ spectrum of PEG-*b*-

PCL with a nominal PCL chain length of 500 repeat units is shown in Figure 28. The ^1H -NMR spectra were used to determine experimentally obtained PCL chain lengths, according to **Equation 8**. The PCL block exhibits a triplet at $\delta = 4.1$ ppm, corresponding to the methylene protons adjacent to the ester group (Figure 28a), a triplet at $\delta = 2.3$ ppm, corresponding to the α -methylene relative to the carbonyl (Figure 28c) and multiplets at $\delta = 1.3$ ppm and 1.6 ppm, corresponding to the methylenes of the chain (Figure 28d & e).²⁰⁸ The ^1H -NMR spectrum of ϵ -caprolactone can be seen in Appendix Figure 8.

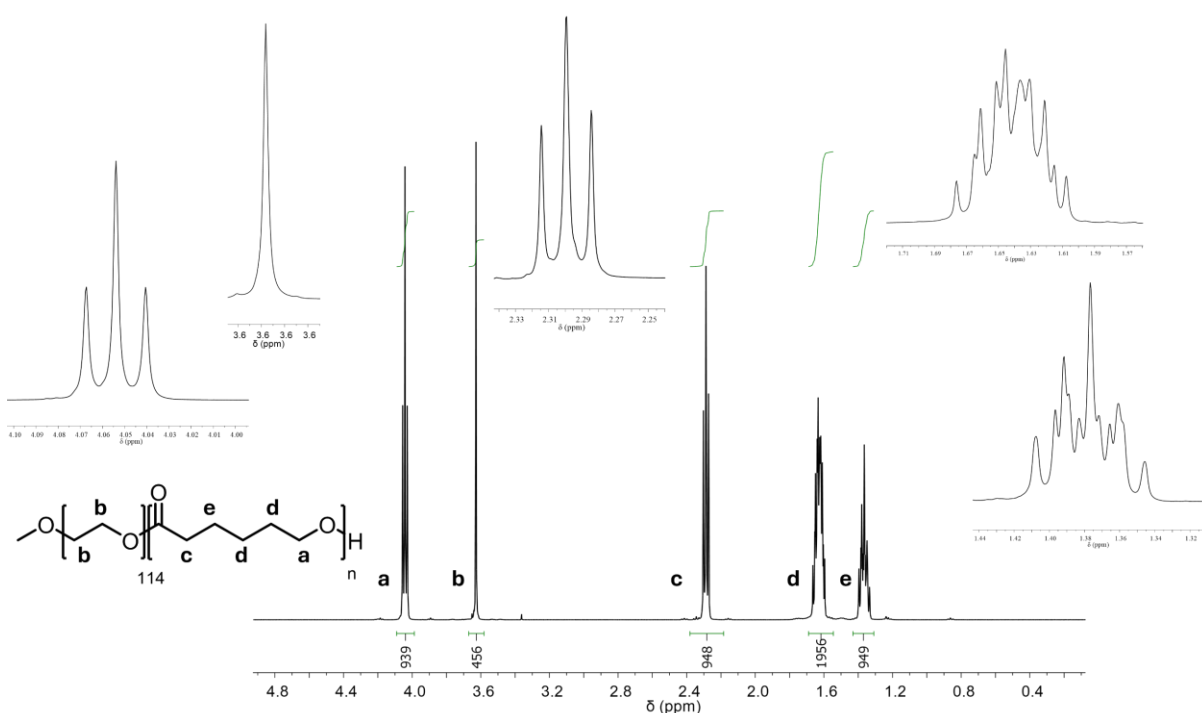


Figure 28. ^1H -NMR Spectrum of $\text{PEG}_{114}\text{-b-PCL}_{500}$ in CDCl_3 .

As with $\text{PEG-}b\text{-PLA}$, the relative intensities of the PCL proton peaks are observed to increase with increasing ϵ -caprolactone:mPEG ratio (Appendix Figure 9). The experimentally obtained PCL block lengths are presented in Table 3. It was proven that control over the degree of polymerisation was achieved, and the obtained

block lengths were in good agreement (95-122%) with the theoretically targeted block lengths.

Table 3. Number average molecular weight (M_n) and corresponding hydrophobic block length of the synthesised PEG-*b*-PCL polymers according to $^1\text{H-NMR}$.

Targeted PCL chain length, n	M_n /kg mol $^{-1}$		PCL block length /number of repeat units	
	Expected	Observed	Expected	Observed
100	16.4	17.8	100	112
150	22.1	25.8	150	183
300	39.2	43.7	300	339
500	62.0	59.6	500	479

Stacked ATR-FTIR spectra of the mPEG macroinitiator, a PCL homopolymer the synthesised PEG-*b*-PCL₁₅₀, enabled identification of characteristic bands for each block (Figure 29, Appendix Table 1). A strong C=O stretching band was observed at $\sim 1722\text{ cm}^{-1}$ for PEG-*b*-PCL₁₅₀, indicative of the formation of the polyesters.²⁰⁸ The characteristic C-O-C PEG ether stretching bands were also present at $\sim 1100\text{ cm}^{-1}$ in PEG-*b*-PCL₁₅₀.²⁰²

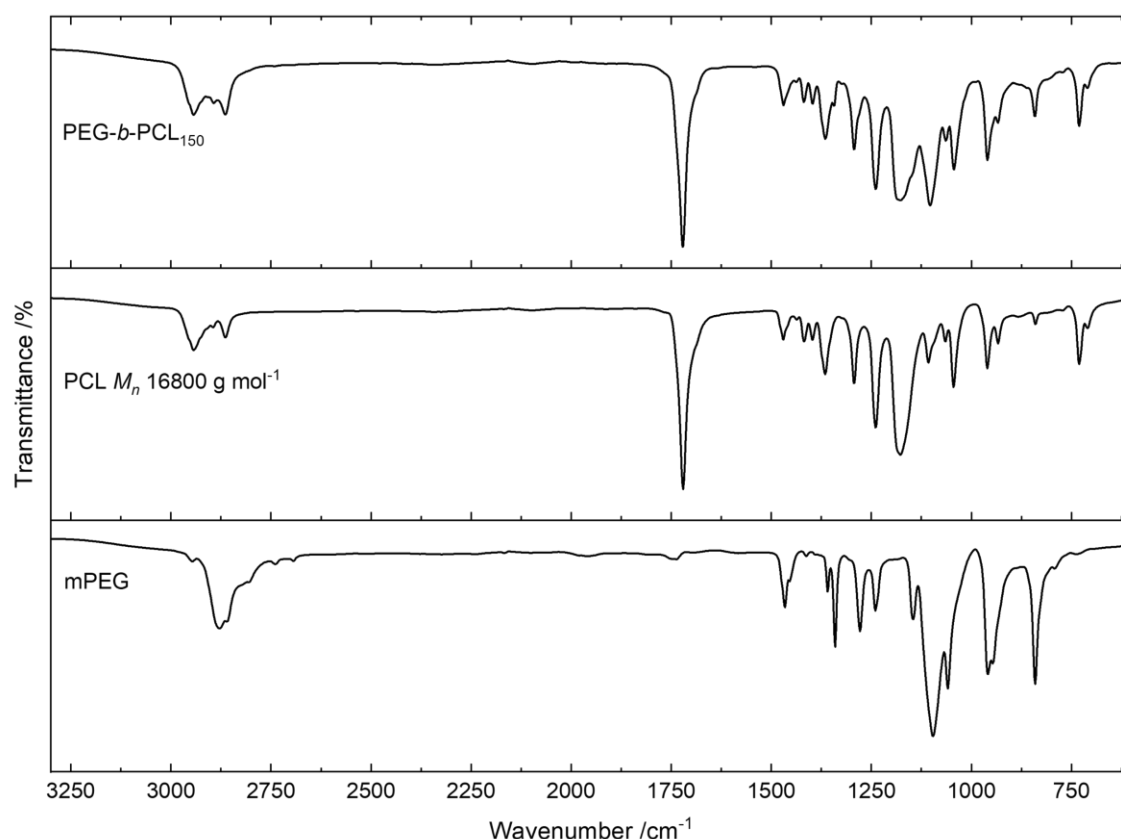


Figure 29. ATR-FTIR spectra of the mPEG macroinitiator, PCL with a M_n of 16800 g mol⁻¹ (purchased from Sigma-Aldrich, Germany) and the synthesised PEG-*b*-PCL₁₅₀.

The TGA trace of PEG-*b*-PCL₅₀₀ shows a sharp mass loss at ~270 °C, attributed to the thermal degradation of the PCL block (Figure 30).²⁰⁹ PCL is more thermally stable than PLA due to the presence of the tertiary carbon adjacent to the ester group in PLA, which stabilises the carbocation and radical intermediates, facilitating scission and backbiting reactions and resulting in a higher propensity for intramolecular transesterification and depolymerisation of PLA.²¹⁰ PEG degradation is observed in a similar temperature range to that of PEG-*b*-PLA. The PCL block length can also be estimated from the relative mass losses as 609 PCL repeat units, slightly longer than the 500 expected and the 479 repeat units calculated from ¹H-NMR.

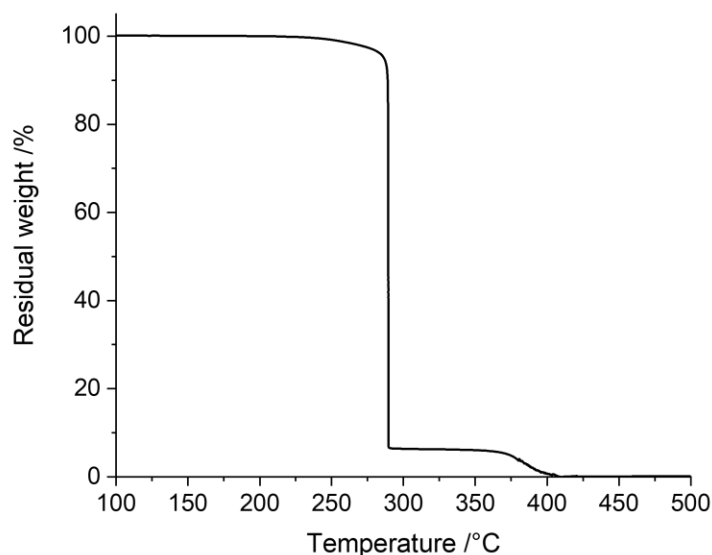


Figure 30. TGA trace of the synthesised PEG-*b*-PCL₅₀₀ under N₂ atmosphere (50 °C min⁻¹).

5.3. Self-assembly into nanostructures

Having established the successful synthesis of the BCPs, their ability to self-assemble into nanoscale structures in mixed media was investigated. The amphiphilic nature of PEG-*b*-PLA and PEG-*b*-PCL enables spontaneous formation of various morphologies, including micelles and vesicles that are able to encapsulate cargo molecules.

Various methods are available for the self-assembly of ABCPs. In general, they rely on microphase separation, in which incompatible blocks separate into distinct domains to reach thermodynamic equilibrium, thereby minimising free energy. The various methods available for self-assembly have been adapted from liposome preparation and include film rehydration, microfluidic emulsification, and nanoprecipitation.^{211–213} While both the former and latter are simple, low-cost techniques, film rehydration generally requires further extrusion to control vesicle size, while nanoprecipitation affords more control.²¹⁴

5.3.1. Size and morphological characterisation

The variation in copolymer concentration is expected to increase the volume fraction of the hydrophobic group relative to the hydrophilic group, increasing the packing parameter, and resulting in a shift from micelle to vesicle formation.¹³⁰ The appearance of self-assembled nanostructures can be observed with the naked eye, as, upon the addition of water, scattering particles cause increased opacity of the suspension. Dynamic Light Scattering (DLS) is typically used to measure the size distribution of assemblies in solution. DLS provides an estimation of the hydrodynamic radius of the particles from the intensity fluctuations of the scattering of incident light by the particles.²¹⁵ The resulting intensity distribution is converted into a number distribution using Mie theory, yielding the number of vesicles within a given size range.²¹⁵

The assemblies formed from solvent shift of PEG-*b*-PLA copolymers had hydrodynamic diameters in the range of 20-300 nm according to DLS measurements (Figure 31). The increasing hydrophobic block length led to an increase in hydrodynamic radius, suggesting a shift from micelles to vesicles.

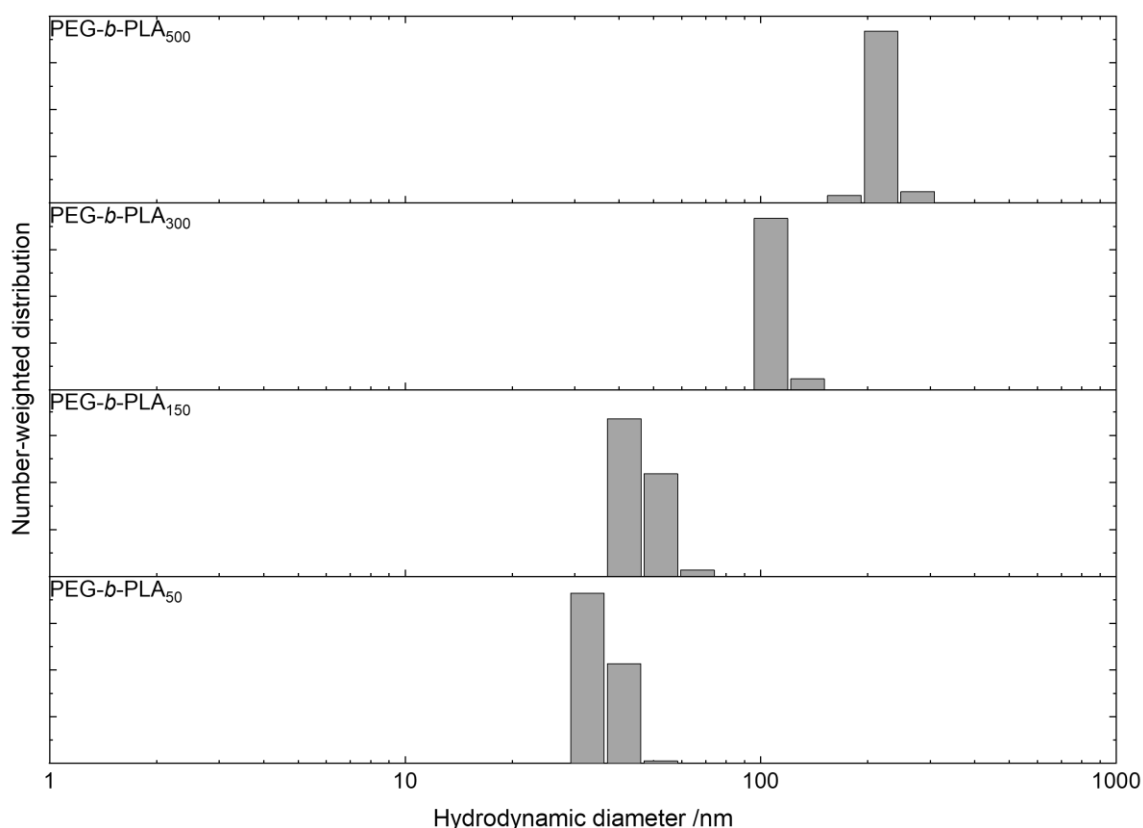


Figure 31. Hydrodynamic diameter distribution by number of PEG-*b*-PLA_x assemblies measured by DLS in Milli-Q water.

This trend was also evident in the FESEM micrographs, which showed assemblies of comparable size to those obtained by DLS, with an increasing particle diameter as the PLA block length increased (Figure 32). FESEM images confirmed the formation of predominantly spherical PEG-*b*-PLA assemblies across all samples. Particle agglomeration was occasionally observed, particularly for copolymers with shorter PLA block lengths, which is commonly attributed to reduced core-forming block hydrophobicity.

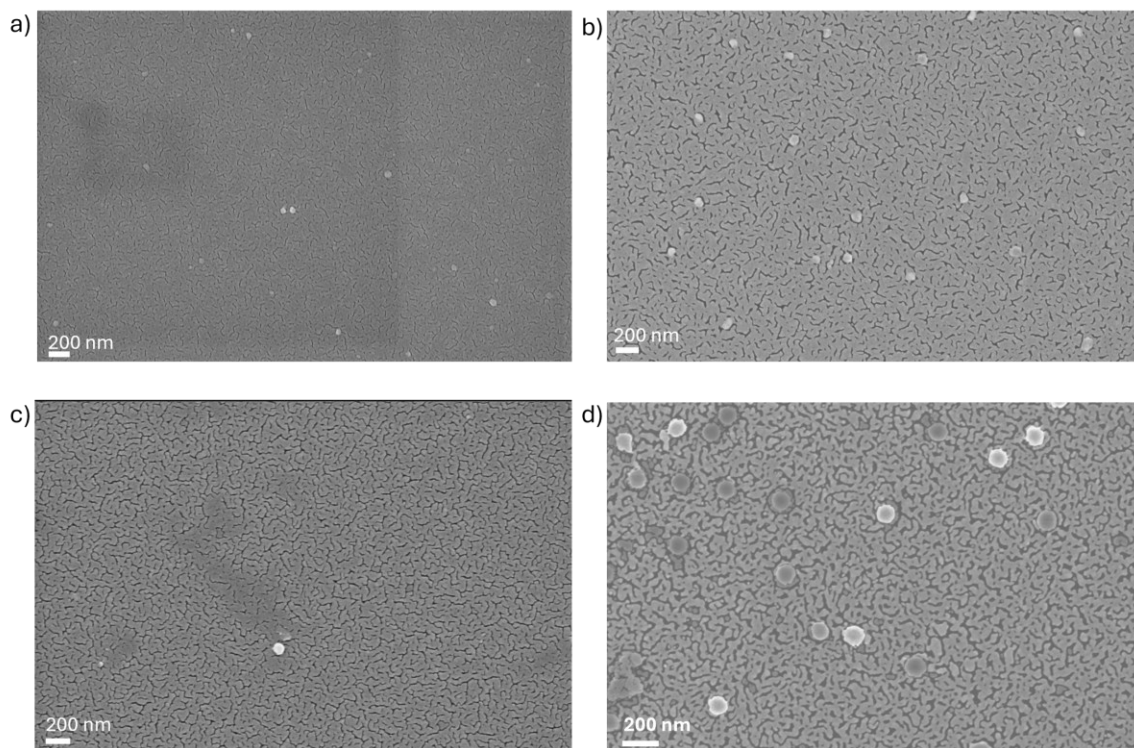


Figure 32. FESEM images of PEG₁₁₄-*b*-PLA_x assemblies, where x is a) 50, b) 150, c) 300 and d) 500. FESEM samples were prepared from a 1 vol% dilution of a vesicle stock solution prepared via solvent shift with Milli-Q water as the aqueous phase.

The diameters of the PEG₁₁₄-*b*-PLA₅₀ and PEG₁₁₄-*b*-PLA₁₅₀ assemblies were determined from FESEM to be 53 ± 13 nm and 73 ± 9 nm, respectively. These values are slightly larger than those obtained by DLS, which can be attributed to drying-induced artefacts during sample preparation for microscopy.^{216,217} Capillary forces resulting in a coffee-ring effect (Appendix Figure 10) and partial particle flattening upon solvent evaporation are known to increase the apparent particle size in microscopy images compared to hydrodynamic diameters measured in solution.^{218,219}

In contrast, the diameters of the PEG₁₁₄-*b*-PLA₃₀₀ and PEG₁₁₄-*b*-PLA₅₀₀ assemblies were measured as 89 ± 6 nm and 154 ± 13 nm, respectively, which are in good agreement with the DLS data. This could be due to the increased structural rigidity and reduced deformability of assemblies with longer PLA blocks, which tend to

form vesicles rather than micelles, leading to thicker membranes and therefore greater morphological stability during drying.^{130,139}

Similarly, the assemblies formed via solvent shift of PEG-*b*-PCL copolymers had average hydrodynamic diameters in the range of 40-250 nm and showed an increase in size with an increase in hydrophobic chain length (Figure 33).

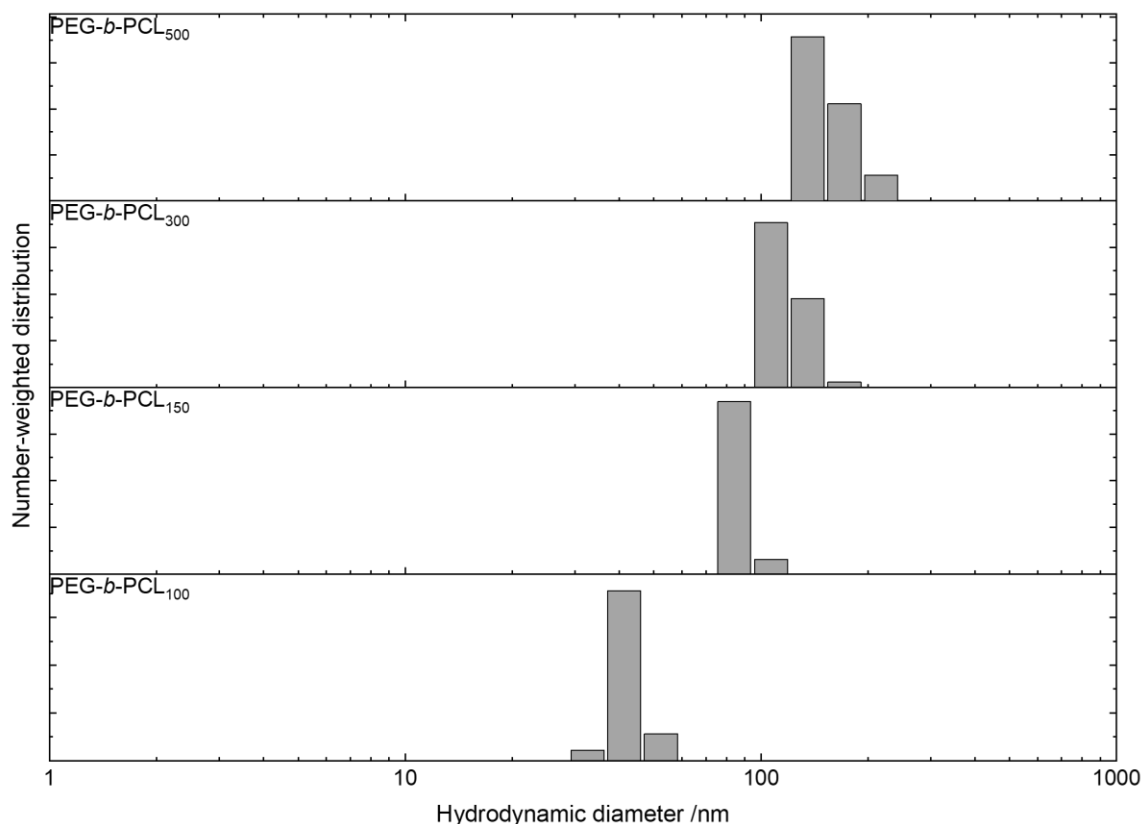


Figure 33. Hydrodynamic diameter distribution by number of PEG-*b*-PCL_x assemblies measured by DLS in Milli-Q water.

The morphology of the PEG-*b*-PCL vesicles was also investigated with FESEM (Figure 34). The diameters of the PEG₁₁₄-*b*-PCL₁₀₀ assemblies were measured as 53 ± 7 nm, which is slightly larger than the hydrodynamic diameter measured via DLS, likely due to the aforementioned drying effects. In contrast, the diameter measured in FESEM for PEG₁₁₄-*b*-PCL₁₅₀ was 70 ± 8 nm, slightly smaller than that measured in DLS, likely due to the agglomeration of the particles, which is also visible in the FESEM image. The diameters of the PEG₁₁₄-*b*-PCL₃₀₀ and PEG₁₁₄-*b*-

PCL₅₀₀ assemblies were measured as 85 ± 11 nm and 111 ± 17 nm, respectively, which are in good agreement with the DLS data.

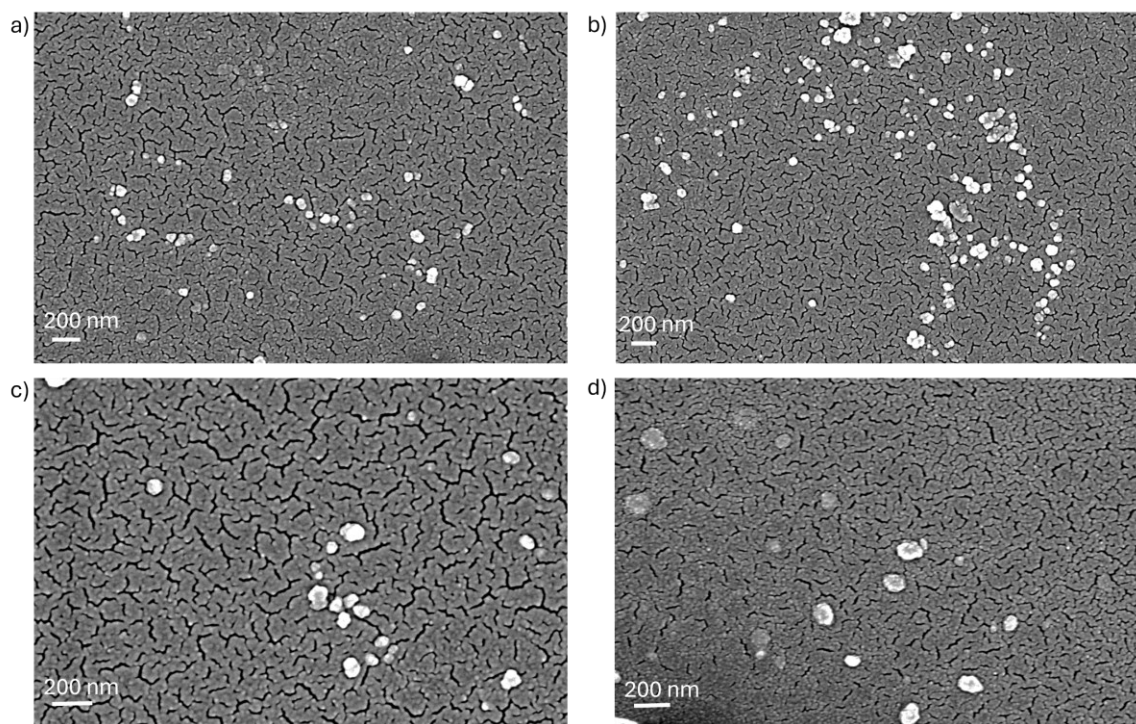


Figure 34. FESEM images of PEG₁₁₄-*b*-PCL_x assemblies, where x is a) 100, b) 150, c) 300 and d) 500. FESEM samples were prepared from a 1 vol% dilution of a vesicle stock solution prepared via solvent shift with Milli-Q water as the aqueous phase.

5.4. Encapsulation of PhB in PEG-*b*-polyester vesicles

The largest assemblies formed from both PEG-*b*-PLA and PEG-*b*-PCL were achieved with the polymers with the longest hydrophobic block lengths. These assemblies are assumed to be vesicles, based on well-reported observations of similar systems, and therefore should contain a large aqueous lumen, facilitating the encapsulation of the largest number of hydrophilic molecules.¹³⁰ The PEG-*b*-PLA₅₀₀ and PEG-*b*-PCL₅₀₀ polymers were therefore self-assembled in the presence of PhB to afford PhB-loaded vesicles.

The resulting suspensions were characterised by DLS, FESEM and TEM. The distributions by number of the suspensions indicated an average hydrodynamic diameter of 230 ± 7 nm for PhB-loaded PEG-*b*-PLA₅₀₀ assemblies and 193 ± 18 nm for PhB-loaded PEG-*b*-PCL₅₀₀ assemblies (Figure 35b). These diameters are significantly larger than those of the water-loaded assemblies (Figure 31 & Figure 33). Similar trends have been observed, for example, by Oz et al., whose poly(2-ethyl-2-oxazoline)-*b*-PLA vesicles increased in size with increasing hydrophilic drug loading.²²⁰

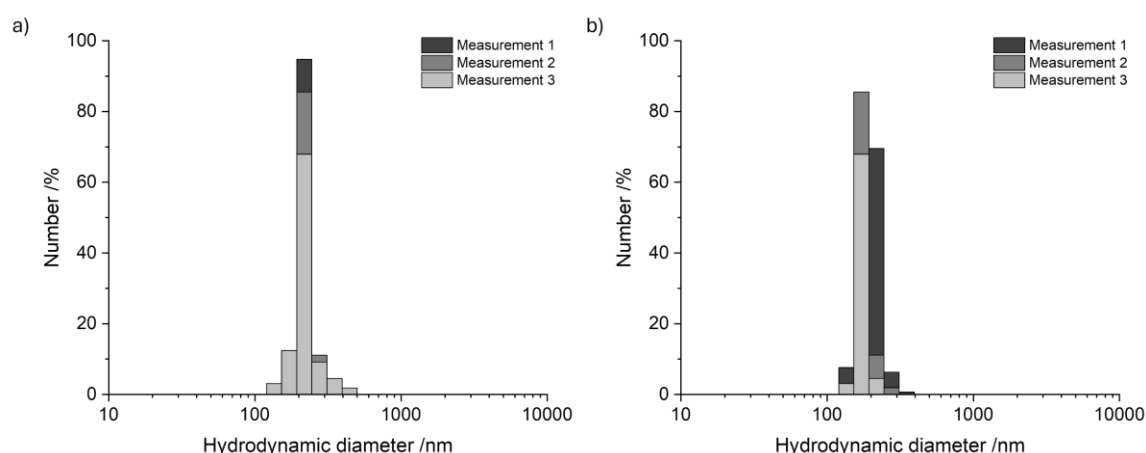


Figure 35. DLS particle size distribution by number of PhB-loaded a) PEG-*b*-PLA₅₀₀ and b) PEG-*b*-PCL₅₀₀ vesicles (1% dilution in Milli-Q water).

The FESEM and TEM images (Figure 36) exhibit spherical features that are likely vesicles, according to the report of Adami et al., who studied very similar PEG-*b*-PLA polymers.²²¹ These round features have diameters consistent with the DLS data. These images also confirm that the spherical shapes are largely retained upon drying, indicating structural stability of the assemblies, which is beneficial for future incorporation into wound dressing materials.

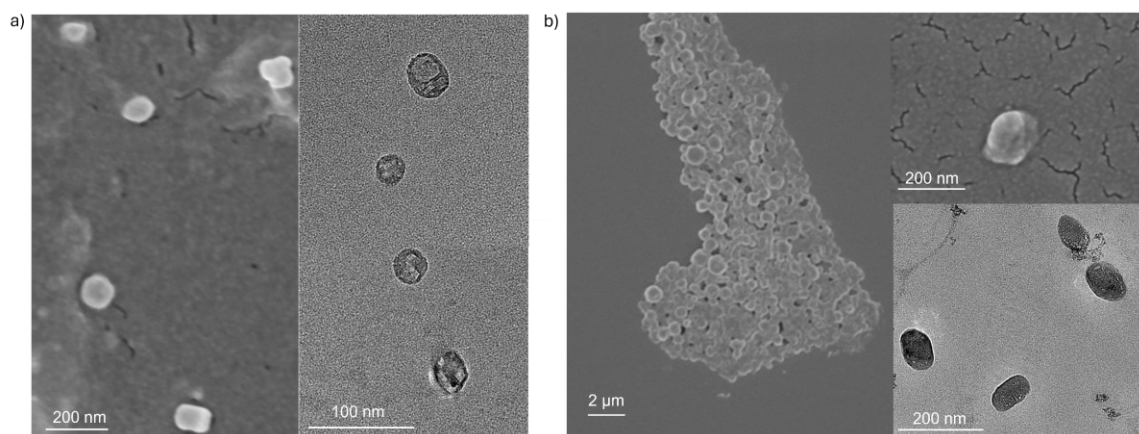


Figure 36. FESEM and TEM images of PhB-loaded a) PEG₁₁₄-*b*-PLA₅₀₀ and b) PEG₁₁₄-*b*-PCL₅₀₀ vesicles (1% v/v dilution in Milli-Q water).

To confirm that PhB is encapsulated, fluorescence microscopy images were acquired. By overlaying the bright field and fluorescence emission images, it is possible to visualise clusters of fluorescent vesicles (Figure 37, Figure 38). Control samples of water-loaded vesicles were non-fluorescent, confirming that the fluorescence is due to the presence of PhB. The fluorescence is isolated to locations where the polymer aggregates are, confirming the PhB is associated with the polymer. However, this method does not enable understanding of the dye's location in the vesicle. Typically, confocal microscopy and analysis of fluorescent lifetimes, which depend on the dye's environment, are utilised.^{222,223} However, due to the lack of photostability of PhB and the autofluorescence of the polymer when excitation wavelengths ~ 500 nm are used, this method is unsuitable. Therefore, the PhB is assumed to be largely associated with the aqueous lumen of the vesicles due to its significant hydrophilicity.

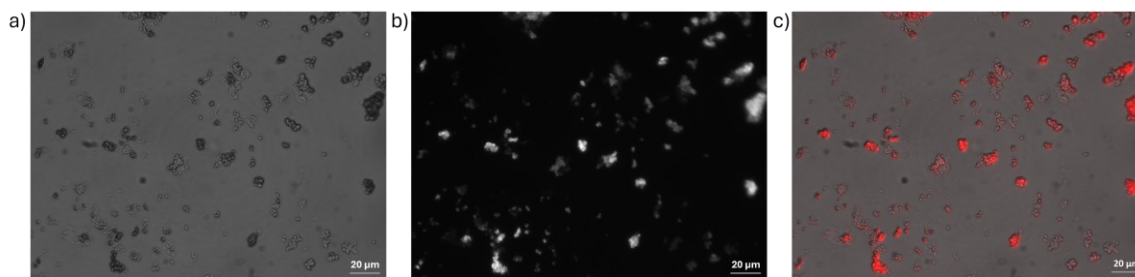


Figure 37. Epifluorescence microscopy image of PhB-loaded PEG-*b*-PLA₅₀₀ vesicles in a) bright field, b) emission resulting from a filter set with $\lambda_{\text{ex}} = 540\text{--}552\text{ nm}$ and $\lambda_{\text{em}} > 590\text{ nm}$ and c) bright field and emission images are merged to localise the PhB. Image shows pseudo-colouring of the fluorescence emission. Scale bars: 20 μm

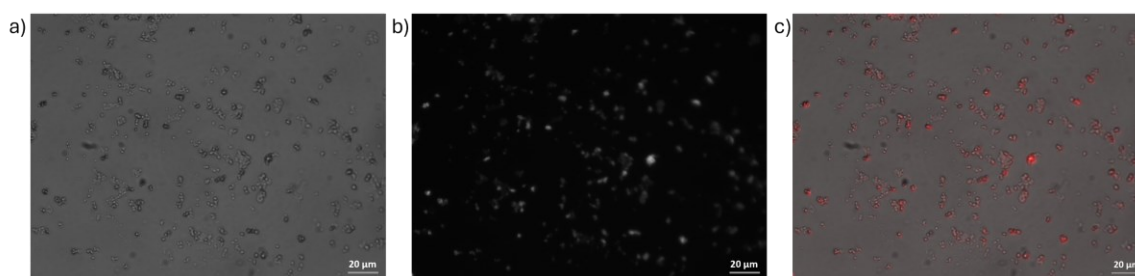


Figure 38. Epifluorescence microscopy image of PhB-loaded PEG-*b*-PCL₅₀₀ vesicles in a) bright field, b) emission resulting from a filter set with $\lambda_{\text{ex}} = 540\text{--}552\text{ nm}$ and $\lambda_{\text{em}} > 590\text{ nm}$ and c) bright field and emission images are merged to localise the PhB. Image shows pseudo-colouring of the fluorescence emission. Scale bars: 20 μm

Attempts to quantify loading with UV-Vis spectroscopy proved difficult as the scattering from the polymer shell in the vesicle suspension prevented any estimation of PhB concentration (Appendix Figure 12). Hence, the absorption spectra were used only to confirm the presence of the dye in the suspension. To estimate the concentration of PhB in the final vesicle suspension, the fluorescence emission of the vesicle suspension (diluted to 5% v/v in Milli-Q water) was measured and compared to the calibration curve of neat PhB in water (Appendix Figure 14). The PhB encapsulated was estimated to be $40 \pm 2.1\text{ }\mu\text{M}$ in PEG₁₁₄-*b*-PCL₅₀₀ vesicles, and $34 \pm 1.6\text{ }\mu\text{M}$ in PEG₁₁₄-*b*-PLA₅₀₀ vesicles, corresponding to encapsulation efficiencies of $4.0 \pm 0.2\%$ and $3.4 \pm 0.1\%$, respectively. Nanocarriers have a high surface-to-volume ratio, resulting in high cargo concentration relative to mass. Therefore, a more concentrated PhB package is created, enabling high local ROS production.²²⁴

5.5. Antimicrobial activity of PhB-loaded carriers

5.5.1. ROS generation

To determine the ability of PhB-loaded vesicles to generate $^1\text{O}_2$, the probe SOSG was incubated with the vesicles, and the impact of increasing irradiation time was investigated (Figure 39). Control experiments performed with water-loaded vesicles confirmed that the signal observed here is indeed due to $^1\text{O}_2$ generation from PhB (Appendix Figure 13), while the increase in fluorescence intensity of the SOSG over time due to self-sensitisation was discussed previously in Chapter 4 (see 4.4.2). The fluorescence intensity of PhB and PhB-loaded vesicles was up to 4-fold higher than the signal from the SOSG itself. This indicates that the irradiation of both neat dye and dye-loaded vesicles generates $^1\text{O}_2$, converting SOSG to SOSG-endoperoxide. Nevertheless, it can be observed that, beyond 15 minutes of irradiation, there is no further apparent $^1\text{O}_2$ production, as has been observed in other systems.²²⁵ This saturation could be consistent with the consumption of all methylantracene moieties or depletion of oxygen in the local environment.²²⁶ It could also be due to the visible light-induced degradation of PhB (see 4.3), resulting in fewer molecules available to sensitise molecular oxygen.¹⁷³

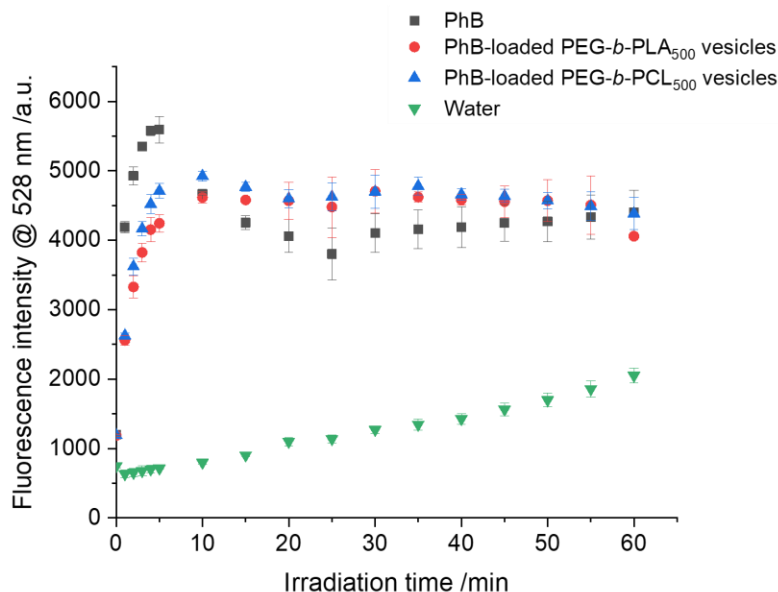


Figure 39. SOSG probe signal to detect $^1\text{O}_2$ production of neat PhB, PhB-loaded vesicle suspensions and water with increasing irradiation times ($\lambda = 520 \text{ nm}$, 4 mW cm^{-2}). The error bars represent the standard deviation ($n = 3$).

5.5.2. Effect of irradiation on vesicle stability

To determine whether the production of $^1\text{O}_2$ from within the vesicle lumen can start to degrade the polymeric bilayer, PhB-loaded PEG-*b*-PLA₅₀₀ vesicles were subjected to green-light irradiation, and their size was investigated by DLS. It was observed that there was no significant change in vesicle diameter even after 20 minutes of irradiation (Figure 40). This indicates that the $^1\text{O}_2$ generated was not sufficient to damage the polymeric bilayer and disassemble the carrier, indicating that PEG-*b*-PLA is sufficiently robust to contain a PS even upon irradiation. However, more subtle morphological changes in the vesicle structure, such as increased permeability, may not be observed via DLS.

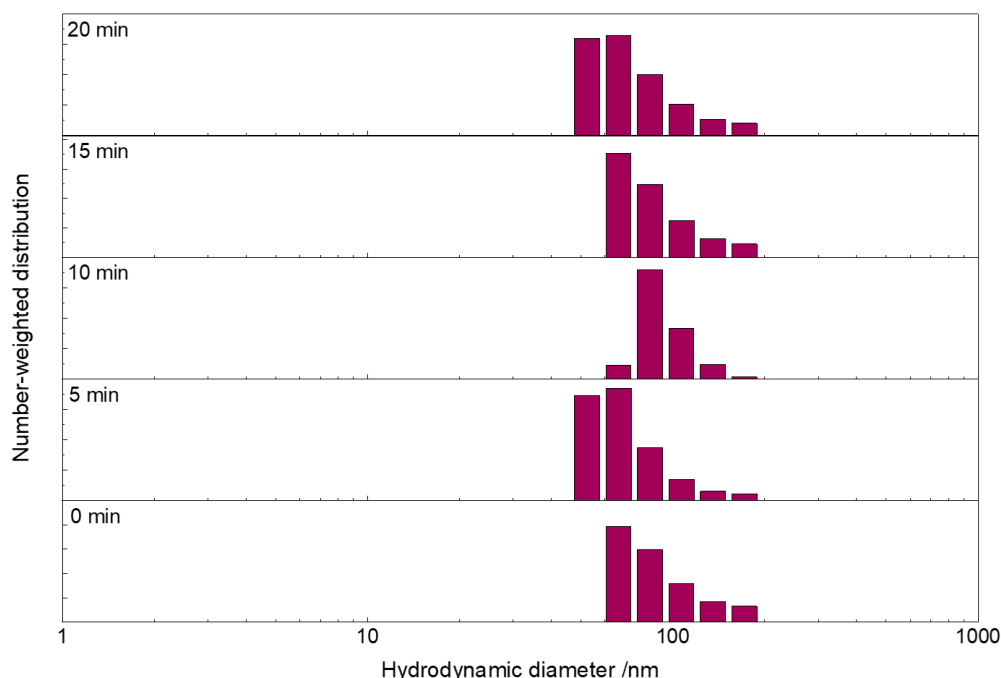


Figure 40. Hydrodynamic diameter distribution by number of PhB-loaded PEG-b-PLA₅₀₀ vesicles upon increasing irradiation (0 to 20 min) with green light.

A subsequent experiment was therefore devised to compare the SOSG fluorescence intensity upon incubation of PhB-loaded PEG-b-PLA₅₀₀ vesicle suspensions that had been pre-irradiated with those that had been kept in the dark. Interestingly, samples which had been pre-irradiated for 15 minutes showed a lower fluorescence intensity upon further irradiation (Figure 41a). If this pre-irradiation caused the leaching of PhB from the vesicles, there would likely be a higher fluorescence of SOSG due to the larger number of PhB available in solution. However, this observation could also be due to the photobleaching of PhB upon irradiation, with the maximum emission observed to decrease to 75% of its original value after 15 minutes of pre-irradiation (Figure 41b).

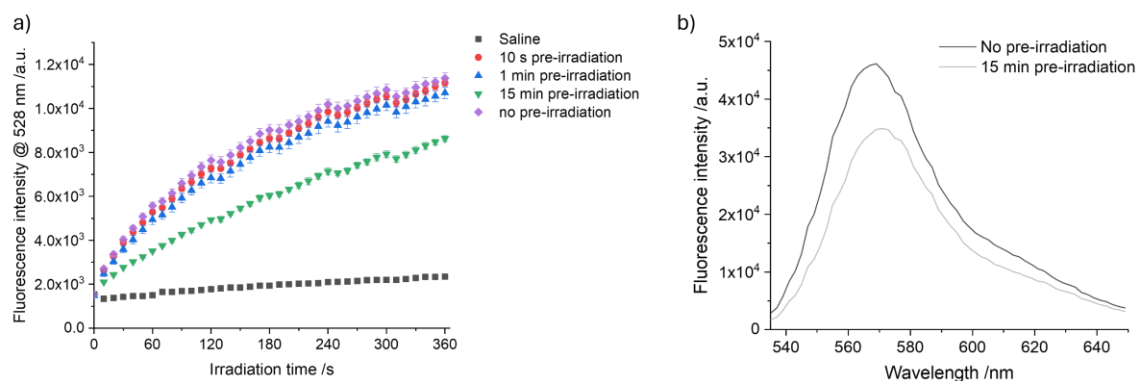


Figure 41. a) Fluorescence intensity of SOSG incubated with PhB-loaded PEG-*b*-PLA₅₀₀ vesicles upon increasing green-light irradiation. b) Fluorescence emission spectra of PhB-loaded PEG-*b*-PLA₅₀₀ vesicles before and after 15 minutes of green-light irradiation.

5.5.3. Antibacterial effect of PhB-loaded vesicles

The PhB-loaded vesicle suspensions were used in antibacterial tests against *S. aureus* lab strain RN4220 and *P. aeruginosa* lab strain ATCC 19660. To ensure all data is comparable, the nominal PhB concentration of both PhB-loaded PEG-*b*-PCL₅₀₀ and PhB-loaded PEG-*b*-PLA₅₀₀ vesicle suspensions was set to 34 μM . Both bacteria were used at a starting concentration of $\sim 10^6$ CFU mL⁻¹, a concentration associated with increased infection risk in wound exudates, and the experiment was performed in the clinically relevant simulated thin wound exudate (STWE).⁵⁸ Both neat PhB and PhB-loaded vesicles were incubated in equal volumes with the bacteria and the number of remaining bacteria after 15 minutes of irradiation (radiant exposure 3.6 J cm⁻²) or dark incubation was determined. Exposure to light irradiation in the absence of vesicles, or in the presence of water-loaded vesicles, had no effect on cultures of *S. aureus* or *P. aeruginosa* (Appendix Figure 15). Without irradiation, neither neat PhB solution nor PhB-loaded vesicle suspensions had any effect on the number of viable *S. aureus* or *P. aeruginosa* (Figure 42). However, upon irradiation of neat PhB and PhB-loaded vesicles, there were no countable *S. aureus* or *P. aeruginosa* colonies observed, representing an LRF of >4.8 for *S. aureus* and >4.7 for *P. aeruginosa*.

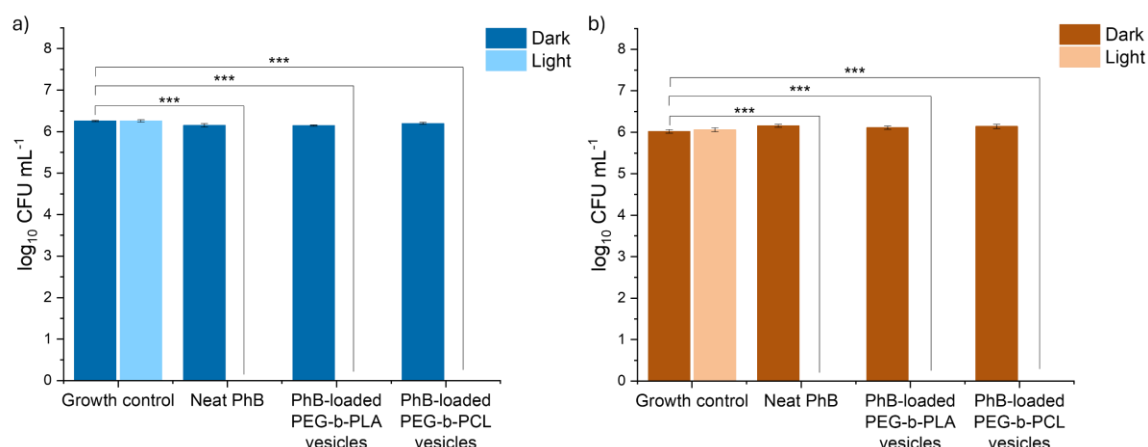


Figure 42. Antimicrobial effectiveness of neat PhB and PhB-loaded vesicles against a) *S. aureus* RN4220 and b) *P. aeruginosa* ATCC 19660 at a concentration of 10^6 CFU mL⁻¹. The samples were irradiated for 15 min ($\lambda = 520$ nm, 4 mW cm⁻²) (Light) or incubated in the dark for 15 min (Dark). The error bars correspond to the standard deviation of biological replicas (n = 3). Asterisks represent significant difference from non-irradiated growth control (***: p < 0.001).

To investigate the extent of the antibacterial activity of the developed PhB-loaded vesicle systems, the experiment was repeated with increasing bacterial concentrations. At a concentration $\sim 10^7$ CFU mL⁻¹ (Figure 43), 15 min irradiation of all PhB-containing samples reduces *S. aureus* load below the limit of detection (LOD), resulting in a LRF of >6.8. In contrast, at this concentration, 15 min irradiation of neither the neat PhB nor the PhB-loaded vesicles were able to completely kill *P. aeruginosa*, with a maximum LRF of 2. A bacterial load of 10^7 CFU mL⁻¹ *P. aeruginosa*, therefore, exceeds the killing capacity of PhB treatment at this concentration and with these irradiation conditions. This can be explained as the same number of PhB molecules (and therefore the same number of ROS) is distributed across 10× more bacterial cells. In addition, the denser bacterial suspension absorbs more light, reducing light penetration and therefore reducing ROS production. While it is possible that longer irradiation times or more concentrated vesicle suspensions could enable killing of higher inoculum concentrations, this data highlights the importance of early detection of *P. aeruginosa* infections for the utilisation of PhB treatment.

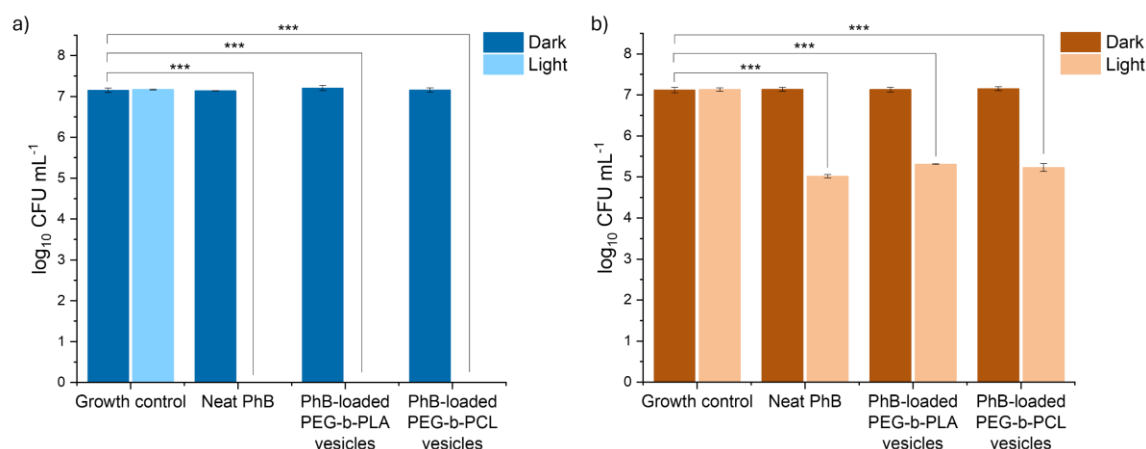


Figure 43. Antimicrobial effectiveness of neat PhB and PhB-loaded vesicles against a) *S. aureus* RN4220 and b) *P. aeruginosa* ATCC 19660 at a concentration of 10^7 CFU mL⁻¹. The samples were irradiated for 15 min ($\lambda = 520$ nm, 4 mW cm⁻²) (Light) or incubated in the dark for 15 min (Dark). The error bars correspond to the standard deviation of biological replicas (n = 3). Asterisks represent significant difference from non-irradiated growth control (***: p < 0.001).

Further increasing the bacteria concentration to $\sim 10^8$ CFU mL⁻¹ shows similar effects to the previous data (Figure 44). While irradiation of PhB and PhB-loaded vesicles completely eradicated *S. aureus*, only a 2 log₁₀ reduction was possible with *P. aeruginosa*. However, this still represents eradication of 9.9×10^8 *P. aeruginosa* cells.

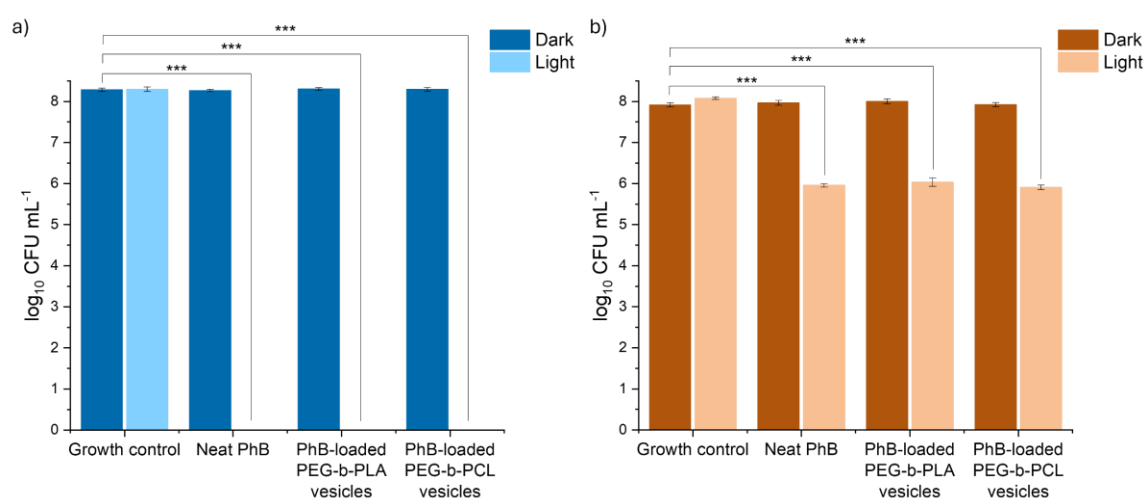


Figure 44. Antimicrobial effectiveness of neat PhB and PhB-loaded vesicles against a) *S. aureus* RN4220 and b) *P. aeruginosa* ATCC 19660 at a concentration of 10^8 CFU mL⁻¹. The samples were irradiated for 15 min ($\lambda = 520$ nm, 4 mW cm⁻²) (Light) or incubated in the dark for 15 min (Dark). The error bars correspond to the standard deviation of biological replicas (n = 3). Asterisks represent significant difference from non-irradiated growth control (***: p < 0.001).

It is advantageous to eradicate both *S. aureus* and *P. aeruginosa*, as polymicrobial wound infections are common; in one study, 27% of wounds were diagnosed with a polymicrobial infection, most commonly *S. aureus* and *P. aeruginosa*.⁶⁶ The susceptibility and antibiotic resistance patterns of different bacterial species and strains differ. Hence, multiple antibiotics would be required to treat one wound. Both PEG-*b*-PLA and PEG-*b*-PCL vesicle suspensions were capable of eradicating both Gram-positive and Gram-negative bacteria. This highlights the ability of both systems to deliver highly concentrated pockets of PhB, which are able to generate $^1\text{O}_2$ and kill bacteria.

A significant advantage of using vesicles rather than neat PhB is their diffusion speed. According to the Stokes–Einstein equation (**Equation 6**), which relates the diffusion coefficient of a particle (D) to its hydrodynamic radius (r), solvent viscosity (η), the Boltzmann constant (k_B) and temperature (T), D for a 200 nm vesicle in water at room temperature is approximately $\sim 2.4 \times 10^{-12} \text{ m}^2 \text{ s}^{-1}$, compared to a PhB molecule which, as a small molecule, is on the order of $10^{-10} \text{ m}^2 \text{ s}^{-1}$.²²⁷ This 100× decrease in diffusion speed results in a higher local concentration of PhB and therefore significant improvements compared to the neat PhB. Following administration to the wound bed, vesicles will remain at the site of infection longer than the neat dye molecules, maintaining a high concentration and facilitating more efficient aPDT. Moreover, it has been reported that the lack of reported microbial resistance to aPDT is likely due to the short interval between PS administration and light irradiation, during which time there is no time to select for resistant strains.²⁵ By minimising contact of PhB with bacteria by containing it inside polymeric vesicles, this approach should therefore limit the ability of the bacteria to develop resistance mechanisms. In addition, the success with a low-cost, low-power, non-coherent LED array enables treatment over a large area due to the wide illumination area relative to a laser light source, without causing damage to healthy tissue.²³ Further work to optimise the concentration of PhB inside the carriers while keeping

this minimal irradiation time could provide an “optimal” system with a low active concentration with the minimum treatment time.

$$D = \frac{k_B T}{6\pi\eta r} \quad \text{Equation 6}$$

The antibacterial action of the vesicles occurs much faster than any release due to enzymatic degradation, which is reported to take hours at high enzyme concentrations.²⁰⁰ This indicates that the PhB is not released at significant concentrations during the experiment, suggesting that internalisation of PhB is not necessary for efficient bacterial killing.

5.6. Pre-Irradiation before incubation

While it has been assumed throughout this thesis that the primary species responsible for bacterial inactivation upon irradiation of PhB and PhB-loaded vesicles was $^1\text{O}_2$, an experiment was devised in which a sample of PhB-loaded PEG-*b*-PLA₅₀₀ vesicles were irradiated in the absence of bacteria for 15 min, then, after a specified waiting period, a *S. aureus* suspension in saline was added. Interestingly, the bacteria were completely eradicated even after waiting 90 s between irradiation and incubation with bacteria (Figure 45). As the lifetime of $^1\text{O}_2$ is on the order of a few microseconds, this prolonged inactivation is likely due to secondary, longer-lived species, such as hydrogen peroxide (H_2O_2). Type I photochemical pathways of PhB upon irradiation can also generate superoxide, which subsequently disproportionates to generate H_2O_2 , which is sufficiently long-lived to persist after irradiation and remain biologically active upon subsequent addition of *S. aureus*.²²⁸ However, repeating this experiment in a more nutrient-rich medium, LB, suggested no longer-lived species remained in the suspension that could kill bacteria (Appendix Figure 16). This is likely due to consumption of the

species by the peptides in the medium, reducing the lifetime of H_2O_2 .²²⁹ The results of this experiment therefore indicate that immobilisation of PhB onto a wound dressing surface may be sufficient to induce bacterial inactivation.

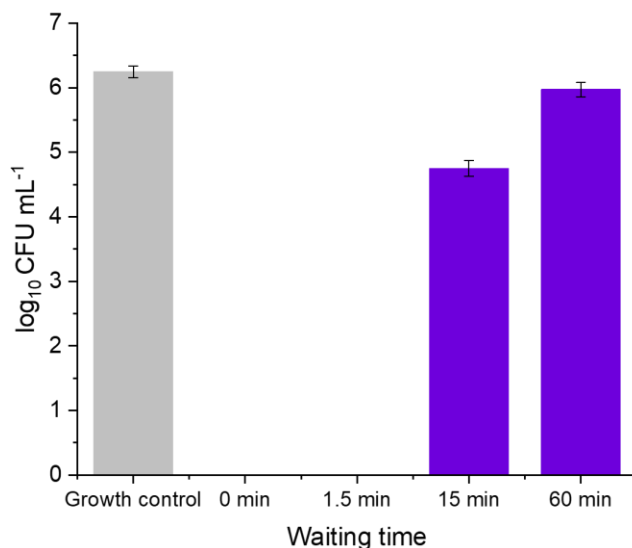


Figure 45. PhB-loaded PEG-*b*-PLA₅₀₀ with *S. aureus* RN4220 10^6 CFU mL^{-1} in saline. Samples were irradiated for 15 min, then a waiting time was observed before adding the bacteria.

5.7. Incorporation of PhB-loaded vesicles into hydrogel supports

To incorporate vesicles into a wound dressing, they would need to be immobilised onto a support matrix. While inkjet printing of PLA nanoparticles onto nonwoven fabrics has been previously demonstrated, the wound care market is increasingly adopting hydrogel-based dressings due to their superior moisture absorption and donation compatibilities, as well as inherent biocompatibility when made of biopolymers.^{230,231}

Hence, as a proof-of-concept for spatially confined delivery, and in collaboration with Dr. Nora Bastida Agote and Dr. Markus Kettel at Paul Hartmann AG, PhB-loaded PEG-*b*-PCL₅₀₀ vesicles were loaded into hydrogel pockets. Biopolymeric

hydrogels crosslinked with an industrial NCO prepolymer were created using a gel coating line. The biopolymers tested included agar, starch, cellulose, alginate, and chitosan. The ratio of biopolymer to crosslinker was adjusted, and the transparency, tacticity, and moisture content of the resulting gels were assessed according to the EN 13726 Primary Wound Dressing Test Methods.

Moulds were utilised to emboss the pockets during crosslinking. Hydrogel pockets made of CS, sodium alginate or carboxymethylcellulose, crosslinked with an NCO-prepolymer, were produced with varied pocket sizes in the millimetre range. This enabled loading of vesicles in microliter quantities per pocket. The vesicles were not observed to leach into the surrounding material, indicating the pockets are sufficient to enclose the suspension (Figure 46). In contrast, neat PhB can be observed to leach from the pocket to the surrounding hydrogel matrix.



Figure 46. Image of loading of CS hydrogel pockets with PhB (top right) and PhB-loaded PEG-*b*-PCL₅₀₀ vesicles (bottom left).

To determine the ability of vesicles to break down, vesicle suspensions were treated with the anionic surfactant sodium dodecyl sulfate (SDS) (Figure 47). After treatment, SDS-polymer interactions led to vesicle disassembly, as confirmed by a ~4-fold decrease in hydrodynamic diameter. This is due to the intercalation of the dodecyl tail of the surfactant into the hydrophobic membrane, rendering the vesicle bilayer unstable. While it is possible that the SDS simply permeates into the vesicle's lumen, this significant size reduction is likely due to disassembly rather than osmotic compression, and matches classical detergent-induced vesicle

solubilisation observed, for example, with liposomes.²³² The released dye also showed a slight “light up” effect, which is visible under UV light (Figure 48). This could therefore be used as an indication of significant bacterial infection, as high bacterial loads secrete higher concentrations of enzymes such as proteases and lipases, which are able to hydrolyse ester bonds and degrade the polymer vesicles.²⁰⁰

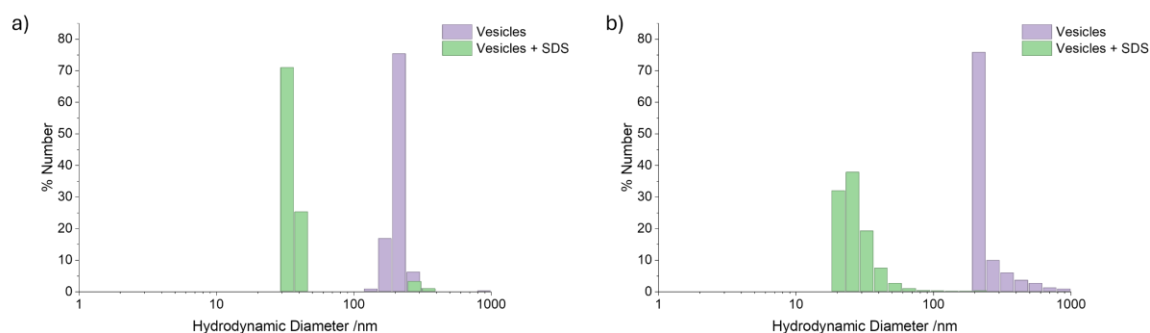


Figure 47. Hydrodynamic diameters of PhB-loaded a) PEG-*b*-PLA₅₀₀ and b) PEG-*b*-PCL₅₀₀ vesicles before and after treatment with SDS (50 mM), measured via DLS.

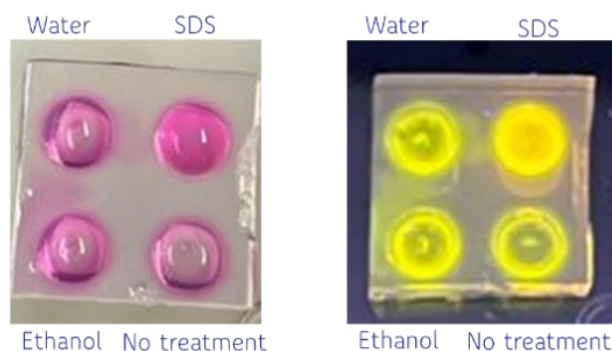


Figure 48. PhB-loaded PEG₁₁₄-*b*-PCL₅₀₀ vesicles in chitosan hydrogel pockets, treated with water (top left), SDS (top right), ethanol (bottom left) or without treatment (bottom right). Left: under white light illumination; right: under UV light.

5.8. Conclusion

This Chapter demonstrates that PEG-*b*-PLA and PEG-*b*-PCL block copolymers can be synthesised with varying hydrophobic block lengths and self-assemble into spherical assemblies, with the size of these assemblies dependent on the

hydrophobic block length. Specifically, PEG-*b*-PLA₅₀₀ and PEG-*b*-PCL₅₀₀ can be self-assembled into vesicles loaded with PhB. These vesicles generate $^1\text{O}_2$ and eradicate both *S. aureus* (LRF > 6.5) and *P. aeruginosa* (LRF > 4.5) upon green light irradiation. Importantly, these vesicles overcome, to some extent, issues of diffusion and localisation of PhB at the site of infection and can be incorporated into hydrogel support matrices to facilitate their use in wound dressing materials. Pre-irradiation experiments, however, suggest that internalisation of the PhB in bacterial cells is not required, due to the presence of longer-lived antimicrobial species. Incorporation of PhB-loaded vesicles into hydrogel pockets is possible, with the vesicles remaining intact unless external chemical agents (SDS) are used to trigger degradation. However, a secondary layer would be required to keep the vesicles hydrated.

5.9. Materials and methods

5.9.1. Materials

Poly(ethylene glycol) monomethyl ether (mPEG₁₁₄, average number molecular weight, M_n , = 5000 g mol⁻¹), L-Lactide ((3S)-*cis*-3,6-dimethyl-1,4-dioxane-2,5-dione, 98%), ϵ -caprolactone (oxepan-2-one, 97%) and stannous octoate (Sn(oct)₂, tin (II) (2-ethylhexanoate, 92.5-100%) were obtained from Sigma-Aldrich (Merck, Germany). All monomers were used as received without further purification. Dichloromethane (DCM, 99.9%) (Fisher Scientific, Germany), tetrahydrofuran (THF, 100%) (VWR, Germany), diethyl ether (99.5%) (Thermo Fisher Scientific, Germany), methanol (99.9%) (Sigma-Aldrich, Merck, Germany), chloroform-d (CDCl₃, 99.8 atom%) (Deutero, Germany) and dimethylsulfoxide-d₆ (DMSO-d₆, 99.8 atom%) (Deutero, Germany) were used as received. Phloxine B (2',4',5',7'-tetrabromo-4,5,6,7-tetrachlorofluorescein disodium salt, dye content $\geq 80\%$), was obtained from Sigma-Aldrich (Merck, Germany). Milli-Q water was drawn from a Millipore

Direct Q8 system with a resistivity of $\geq 18.2 \text{ M}\Omega \text{ cm}$ (Millipore Advantage A10 system, Schwalbach, with Millimark Express 40 filter, Merck, Germany).

Spectra/Por® dialysis membranes (molecular weight cut-off 6-8 kDa) were purchased from Carl Roth GmbH (Germany). Acrylic cuvettes and transparent flat-bottom polystyrene 96-well plates were purchased from Sarstedt (Germany), and cell culture 96 black half-area μ -clear well plates and 384-well plates from Greiner Bio-One (Germany). Glass NMR tubes (Boro400–5–7) were purchased from Deutero GmbH (Germany).

Lysogeny broth (LB medium, 10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 10 g L^{-1} NaCl, pH 7.0 ± 0.2) and LB agar (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 10 g L^{-1} NaCl, 15 g L^{-1} agar-agar, pH 7.0 ± 0.2) were purchased from Carl Roth GmbH (Germany). Phosphate buffer solution (PBS, pH 7.4) was prepared by dissolving a PBS tablet (Sigma Aldrich, Merck, Germany) in 200 mL Milli-Q water. Simulated thin wound exudate (STWE) was prepared by dissolving sodium chloride (NaCl, 0.8298%, Sigma Aldrich, Merck, Germany) and calcium chloride (CaCl_2 , 0.0368%, AppliChem, Germany) in Milli-Q water. Singlet Oxygen Sensor Green (SOSG) was purchased from Thermo Fisher Scientific (Germany), and Tris buffer (100 mM, pH 7.4) from Millipore (MA, USA).

Pseudomonas aeruginosa lab strain ATCC 19660 (isolated from sepsis in Lima, Peru, and purchased from LGC Standard GmbH (Germany)) and *Staphylococcus aureus* lab strain RN4220 (ATCC 35556; originally derived from NCTC 8325-4 via UV and chemical mutagenesis, purchased from Leibniz-Institute DSMZ GmbH (Germany)) were used in this study. All bacteria were grown in LB medium at 37°C .

5.9.2. Ring-opening polymerisation (ROP) of lactones

The ROP of the lactones L-lactide and ϵ -caprolactone was performed in the bulk according to previous methods.^{199,200} Prior to synthesis, all solid reagents were dried overnight *in vacuo* (5 mbar, rt), and all glassware was dried overnight at 80°C . All

syntheses were performed on a Schlenk line. Glassware was built up hot, ensuring sufficient greasing, then was cycled onto the line 3× with heating under vacuum, followed by backfilling with argon.²³³ The addition of reactants was performed under constant argon flux.

Poly(ethylene glycol)-*block*-poly(lactic acid) (PEG-*b*-PLA)

PEG₁₁₄-*b*-PLA_x was synthesised via ROP of L-lactide, with mPEG₁₁₄ as macroinitiator, and Sn(oct)₂ as catalyst. Molar ratios were adjusted according to the desired PLA chain length. Briefly, with amounts corresponding to PEG₁₁₄-*b*-PLA₅₀₀, mPEG₁₁₄ (5 kg mol⁻¹, 100 μmol) was added to Sn(oct)₂ (0.1 mL) at 80 °C under stirring. After heating to 130 °C, L-lactide (25 mmol) was added. The mixture was stirred for 30 min, then cooled to room temperature. The resulting polymer was dissolved in DCM and precipitated in excess ice-cold diethyl ether (>10× volume of DCM) to remove unreacted mPEG₁₁₄ and L-lactide. The reprecipitation was repeated 5× in total, and the final white solid product was dried overnight (20 °C, 5 mbar).

Poly(ethylene glycol)-*block*-poly(caprolactone) (PEG-*b*-PCL)

PEG₁₁₄-*b*-PCL_x was synthesised via ROP of ε-caprolactone, with mPEG₁₁₄ as macroinitiator, and Sn(oct)₂ as catalyst. Molar ratios were adjusted according to the desired PCL chain length. Briefly, with amounts corresponding to PEG₁₁₄-*b*-PCL₅₀₀, mPEG₁₁₄ (5 kg mol⁻¹, 100 μmol) was added to ε-caprolactone (50 mmol) at room temperature under stirring. After 15 minutes, the mixture was heated to 110 °C. Sn(oct)₂ was added and the mixture was stirred for 20 h. The product was then cooled to room temperature. The resulting polymer was dissolved in chloroform and precipitated in ice-cold methanol to remove unreacted ε-caprolactone and mPEG₁₁₄. The reprecipitation was repeated 5× in total, and the final white solid product was dried overnight (20 °C, 5 mbar).

5.9.3. ^1H NMR spectroscopy

The polymers were characterised via ^1H -NMR spectroscopy in CDCl_3 or DMSO-d_6 for short-chain polymers. The spectra were referenced to the residual proton signal of the solvent ($\delta(^1\text{H})$: CHCl_3 = 7.25 ppm, DMSO-d_6 = 2.50 ppm). Spectra were recorded on a Jeol ECZ 500 MHz spectrometer at room temperature by Dr. rer. nat. Thomas Paululat. The raw data was processed with MestReNova (Version 6.0.2-5475). The observed chemical shifts (δ) are stated in ppm relative to the solvent signal, and J values are given in Hz.

PEG-*b*-PLA₁₀₀ δ_{H} (500 MHz; CDCl_3): 7.26 (1 H, s, CHCl_3), 5.16 (82 H, q, ^3J 7.1, $\text{CH}_3\text{CH}(\text{C}=\text{O})\text{O}$ (PLA)), 3.64 (456 H, s, $\text{OCH}_2\text{CH}_2\text{O}$ (PEG)), 3.36 (3 H, s, OCH_3 (PEG-OMe)), 1.56 (267 H, d, ^3J 7.1, $\text{CH}_3\text{CH}(\text{C}=\text{O})\text{O}$ (PLA))

PEG-*b*-PLA₁₅₀ δ_{H} (500 MHz; CDCl_3): 7.26 (1 H, s, CHCl_3), 5.16 (134 H, q, ^3J 7.1, $\text{CH}_3\text{CH}(\text{C}=\text{O})\text{O}$ (PLA)), 3.64 (456 H, s, $\text{OCH}_2\text{CH}_2\text{O}$ (PEG)), 3.36 (3 H, s, OCH_3 (PEG-OMe)), 1.57 (429 H, d, ^3J 7.1, $\text{CH}_3\text{CH}(\text{C}=\text{O})\text{O}$ (PLA))

PEG-*b*-PLA₃₀₀ δ_{H} (500 MHz; CDCl_3): 7.26 (1 H, s, CHCl_3), 5.16 (310 H, q, ^3J 7.1, $\text{CH}_3\text{CH}(\text{C}=\text{O})\text{O}$ (PLA)), 3.64 (456 H, s, $\text{OCH}_2\text{CH}_2\text{O}$ (PEG)), 3.36 (3 H, s, OCH_3 (PEG-OMe)), 1.56 (952 H, d, ^3J 7.1, $\text{CH}_3\text{CH}(\text{C}=\text{O})\text{O}$ (PLA))

PEG-*b*-PLA₄₀₀ δ_{H} (500 MHz; CDCl_3): 7.26 (1 H, s, CHCl_3), 5.16 (406 H, q, ^3J 7.1, $\text{CH}_3\text{CH}(\text{C}=\text{O})\text{O}$ (PLA)), 3.64 (456 H, s, $\text{OCH}_2\text{CH}_2\text{O}$ (PEG)), 3.36 (3 H, s, OCH_3 (PEG-OMe)), 1.57 (1258 H, d, ^3J 7.1, $\text{CH}_3\text{CH}(\text{C}=\text{O})\text{O}$ (PLA))

PEG-*b*-PLA₅₀₀ δ_{H} (500 MHz; CDCl_3): 7.26 (1 H, s, CHCl_3), 5.16 (483 H, q, ^3J 7.1, $\text{CH}_3\text{CH}(\text{C}=\text{O})\text{O}$ (PLA)), 3.64 (456 H, s, $\text{OCH}_2\text{CH}_2\text{O}$ (PEG)), 3.36 (3 H, s, OCH_3 (PEG-OMe)), 1.57 (1707 H, d, ^3J 7.1, $\text{CH}_3\text{CH}(\text{C}=\text{O})\text{O}$ (PLA))

PEG-*b*-PCL₁₀₀ δ_{H} (500 MHz; CDCl_3): 7.26 (1 H, s, CHCl_3), 4.0-4.1 (222 H, t, ^3J 6.8, CH_2O (PCL)), 3.64 (456 H, s, $\text{OCH}_2\text{CH}_2\text{O}$ (PEG)), 3.36 (3 H, s, OCH_3 (PEG-OMe)), 2.30 (225 H, t, ^3J 7.5, CH_2CO (PCL)), 1.65 (451 H, m, ^3J 7.5, CH_2CH_2 (PCL)), 1.35 (223 H, m, ^3J 7.5, CH^2 (PCL))

PEG-*b*-PCL₁₅₀ δ_{H} (500 MHz; CDCl_3): 7.26 (1 H, s, CHCl_3), 4.0-4.1 (354 H, t, ^3J 6.8, CH_2O (PCL)), 3.64 (456 H, s, $\text{OCH}_2\text{CH}_2\text{O}$ (PEG)), 3.36 (3 H, s, OCH_3 (PEG-OMe)), 2.30 (358 H, t, ^3J 7.5, CH_2CO (PCL)), 1.65 (750 H, m, ^3J 7.5, CH_2CH_2 (PCL)), 1.35 (366 H, m, ^3J 7.5, CH^2 (PCL))

PEG-*b*-PCL₃₀₀ δ_{H} (500 MHz; CDCl_3): 7.26 (1 H, s, CHCl_3), 4.0-4.1 (663 H, t, ^3J 6.8, CH_2O (PCL)), 3.64 (456 H, s, $\text{OCH}_2\text{CH}_2\text{O}$ (PEG)), 3.36 (3 H, s, OCH_3 (PEG-OMe)), 2.30 (666 H, t, ^3J 7.5, CH_2CO (PCL)), 1.65 (1320 H, m, ^3J 7.5, CH_2CH_2 (PCL)), 1.35 (663 H, m, ^3J 7.5, CH^2 (PCL))

OMe)), 2.30 (670 H, t, ^3J 7.5, CH_2CO (PCL)), 1.65 (1393 H, m, ^3J 7.5, CH_2CH_2 (PCL)), 1.35 (667 H, m, ^3J 7.5, CH^2 (PCL))

PEG-*b*-PCL₅₀₀ δ_{H} (500 MHz; CDCl_3): 7.26 (1 H, s, CHCl_3), 4.0-4.1 (929 H, t, ^3J 6.8, CH_2O (PCL)), 3.64 (456 H, s, $\text{OCH}_2\text{CH}_2\text{O}$ (PEG)), 3.36 (3 H, s, OCH_3) (PEG-OMe)), 2.30 (935 H, t, ^3J 7.5, CH_2CO (PCL)), 1.65 (1974 H, m, ^3J 7.5, CH_2CH_2 (PCL)), 1.35 (940 H, m, ^3J 7.5, CH^2 (PCL))

The chain lengths of the hydrophobic blocks were also determined via ^1H -NMR spectroscopy. The integral of the mPEG protons at 3.64 ppm was normalised to 456 protons, assuming a M_n of 5,000 g mol $^{-1}$. The intensities of the hydrophobic proton peaks were compared to those of the PEG protons (CH_2CH_2 signal at 3.64 ppm) as per **Equation 7** (PEG-*b*-PLA) or **Equation 8** (PEG-*b*-PCL).

$$\frac{cru_{\text{PLA}}}{cru_{\text{PEG}}} = \frac{I_{\text{PLA}} N_{\text{PEG}}}{I_{\text{PEG}} N_{\text{PLA}}} = \frac{(I_{5.2\text{ppm}} + I_{1.6\text{ppm}}) \frac{4}{4}}{I_{3.64\text{ppm}}} = \frac{(I_{5.2\text{ppm}} + I_{1.6\text{ppm}})}{I_{3.64\text{ppm}}} \quad \text{Equation 7}$$

$$\frac{cru_{\text{PCL}}}{cru_{\text{PEG}}} = \frac{I_{\text{PCL}} N_{\text{PEG}}}{I_{\text{PEG}} N_{\text{PCL}}} = \frac{(I_{4.1\text{ppm}} + I_{2.3\text{ppm}} + I_{1.7\text{ppm}} + I_{1.4\text{ppm}}) \frac{4}{10}}{I_{3.64\text{ppm}}} \quad \text{Equation 8}$$

5.9.4. Gel permeation chromatography (GPC)

The copolymer polydispersity index (PDI), number average molecular weight (M_n) and weight average molecular weight (M_w) were determined via GPC (PSS-SECcurity system Agilent Technologies 1260 infinity, equipped with a PSS GRAM 100A column and a 10 μm particle size pre-column), with polystyrene used as the standard (Polymer Standard Service, Mainz, Germany) and tetrahydrofuran (THF) as the eluent. The flow rate was 1 mL min $^{-1}$ and the refractive index was detected. GPC measurements were performed by Dr. rer. nat. Maximilian Meier. PLA chain lengths were calculated from the obtained M_n values as per **Equation 9**.

$$cru_{\text{PLA}} = \frac{M_n(\text{BCP}) - M_n(\text{PEG}_{114})}{M_n(\text{PLA}_{83})/83} \quad \text{Equation 9}$$

5.9.5. Thermogravimetric analysis (TGA)

Thermogravimetric analysis (TGA) of synthesised polymers was performed on a TGA Q50 V6.7 Build 203 (Universal V4.4A, TA Instruments). The sample was dried overnight at 5 mbar, filled into a platinum crucible, placed in the auto sampler of the device, and equilibrated at 50 °C under a nitrogen atmosphere. After equilibration, the temperature was increased (50 °C min⁻¹) to 500 °C. The gas was then changed to oxygen to oxidise remaining organic residues and the temperature was increased (50 °C min⁻¹) from 500 to 850 °C. TGA measurements were performed by Dr. Prasamsha Panta.

5.9.6. Attenuated total internal reflection Fourier transform infrared spectroscopy (ATR-FTIR)

Attenuated total reflection infrared (ATR-IR) spectra were recorded on a Tensor 27 Fourier transform infrared spectrometer equipped with a diamond crystal (Vertex Neo R, Bruker Optik GmbH, Germany) in the 500 to 4000 cm⁻¹ range (non-polarised) with a spectral resolution of 4 cm⁻¹.

5.9.7. Self-assembly via nanoprecipitation

PEG-*b*-PLA and PEG-*b*-PCL nanoassemblies were prepared via the solvent-shift (or nanoprecipitation) method. The aqueous phase was added at a rate of 0.5 mL min⁻¹ using a syringe pump (AL-400, WPI, US) to a stirring solution containing an equal volume of BCP (3 wt% in THF). For the encapsulation of PhB, a 1 mM solution in Milli-Q was used as the aqueous phase. The resulting milky suspension was stirred for 18 h at room temperature, then a 10× excess of Milli-Q water was added to freeze the structures. The turbid mixture was dialysed (MWCO = 6-8 kDa) for 7 days against Milli-Q water, exchanging the surrounding water 3-4 times per day.

Cargo concentration and encapsulation efficiency were determined via fluorescence spectroscopy. The emission intensity of blank vesicles was subtracted from PhB-loaded vesicles, and the PhB concentration estimated by

comparison with the calibration curve. Encapsulation efficiency was calculated as the ratio of the concentrations of encapsulated to initial PhB concentration.

5.9.8. Dynamic light scattering (DLS) measurements

To determine average hydrodynamic diameter and size distributions, the vesicle suspensions were measured via Dynamic Light Scattering (DLS) using a Wyatt DynaPro Plate Reader III ($\lambda = 818.3$ nm, detector angle 150° , Version 3.0.2.1). Vesicle stock solutions were diluted to 1 wt% in Milli-Q water. 30 μ L aliquots were measured in triplicate in a 384-well plate at 25 $^\circ$ C (5 s/acquisition, 5 acquisitions/measurement). A Gaussian fit (OriginPro 2018) was used to calculate the mean hydrodynamic diameter from the number-weighted results of three independent measurements (three samples).

5.9.9. Field emission scanning electron microscopy (FESEM)

The morphology of the dried assemblies was observed via FESEM (Zeiss Ultra 55cv, Zeiss, Oberkochen, Germany) by drop-casting a diluted vesicle suspension (1% v/v) onto silicon wafers pre-cleaned with chloroform, ethanol, water, and UV-ozone. To generate a conductive surface, samples were sputtered with gold for 30 s, resulting in a $\sim 5.0 \pm 0.5$ nm gold layer. FESEM measurements were collected using the InLens secondary electron detector, with an operation voltage of 5 kV. FESEM images were recorded by M.Sc. Aysha Awan and M.Sc. Winny Chelangat. The images were analysed using ImageJ, and diameters calculated as an average of ≥ 20 particles (software version 1.53r).

5.9.10. Transmission electron microscopy (TEM)

Transmission Electron Microscopy (TEM, FEI Talos F200X) images of samples drop-cast onto copper grids (mesh 400) with ultra-thin carbon film (< 3 nm) supported by lacy-carbon-film (Plano GmbH, Germany) were recorded using an acceleration voltage of 80-200 kV. TEM imaging was performed by M.Sc. Max Müller. The images were analysed using ImageJ.

5.9.11. Epifluorescence microscopy

Epifluorescence microscopy was used to visualise the fluorescence of PhB in the vesicle suspensions using an Axiovert 135 microscope equipped with an AxioCamMRm and Zen 2.3lite software and a filter set with λ_{ex} = 540–552 nm and λ_{em} > 590 nm (Carl Zeiss MicroImaging GmbH, Jena, Germany). The camera exposition time was 80 ms in brightfield and 2000 ms with the filter set.

5.9.12. Irradiation experiments

Irradiation was performed using a home-built device (Fig. S5). A flat-bottom polystyrene 96-well plate (Sarstedt, Germany) was half-filled with 48 green LED bulbs (3 V, 20 mA, type 15505, λ_{max} = 520 nm, 4 mW cm⁻²). This well plate was placed directly on top of the irradiated plate. Unless stated otherwise, after a 15-minute incubation period, samples were irradiated thrice for 5 minutes, with 5 minutes in the dark (covered in aluminium foil) between each exposure. The irradiation was carried out at room temperature (22 °C). The radiant exposure was calculated using **Equation 10**.

$$\begin{aligned} \text{radiant exposure (J/cm}^2\text{)} \\ = \text{irradiance (W/cm}^2\text{)} \times \text{exposure time (s)} \end{aligned} \qquad \text{Equation 10}$$

5.9.13. Bacterial culture preparation

A sterile loop was used to streak glycerol stocks onto LB agar plates, which were incubated at 37 °C for 18 h. To prepare overnight bacterial cultures, a single colony was picked from an LB agar plate and used to inoculate 5 mL of LB medium, followed by incubation at 37 °C for 18 h while shaking at 200 rpm. Bacterial pellets were isolated by centrifugation of the overnight culture (5000×g, 5 min, 4 °C) and removal of the supernatant. The pellets were washed twice with STWE, and the optical density at 600 nm (OD₆₀₀) adjusted in STWE to 0.6 (*P. aeruginosa*) or 1.2 (*S. aureus*), corresponding to ~10⁸ CFU mL⁻¹. This solution was used as prepared or

further diluted 10 $\times$ or 100 $\times$ in STWE to obtain final working concentrations of $\sim 10^7$ and 10^6 CFU mL $^{-1}$.

5.9.14. Detection of singlet oxygen production

The fluorescent probe singlet oxygen sensor green (SOSG) was used to detect $^1\text{O}_2$. A 5 mM stock solution of SOSG was prepared by dissolving the contents of a vial (100 μg) in 33 μL methanol. Aliquots of 100 μM were prepared in Tris buffer and stored in the dark at -20 $^\circ\text{C}$. To determine singlet oxygen production, 45 μL of the sample was mixed with 45 μL of medium (Milli-Q or bacterial culture in STWE) in a well of a black 96-well plate. Then, 10 μL of SOSG (100 μM in methanol) was added to reach a final SOSG concentration of 10 μM . Fluorescence emission intensity ($\lambda_{\text{ex}} = 504 \pm 5$ nm, $\lambda_{\text{em}} = 528 \pm 5$ nm) was measured (Tecan Safire (F129013), Tecan, Switzerland) after regular intervals of light irradiation. Each sample was measured in triplicate.

5.9.15. Antimicrobial photodynamic therapy with planktonic bacteria

In a 96-well plate, 25 μL bacteria ($\sim 10^6$ CFU mL $^{-1}$) were incubated with 25 μL PhB-loaded vesicles or a neat PhB solution (34 μM). The final volume in each well was a 2 $\times$ dilution of both the treatment and the bacteria suspension. Each sample was tested in technical triplicate. The samples were incubated in the dark at room temperature for 15 minutes, then irradiated. Non-irradiated samples were kept in the dark for the same time duration. To evaluate the aPDT effect, each technical replicate was serially diluted (10 $\times$) in PBS and 3 $\times$ 10 μL was plated for each dilution step on LB agar plates, resulting in a limit of detection of 34 CFU mL $^{-1}$. Plates were incubated for 16 h at 37 $^\circ\text{C}$, then bacterial colonies were counted. From this count, the log $_{10}$ CFU mL $^{-1}$ and the LRF were calculated. For the LRF, the non-irradiated bacteria suspension was taken as the control. The arithmetic mean and standard deviation were calculated from 3 independently performed experiments (biological

triplicate). Statistical significance was determined using one-way analysis of variance (ANOVA) and the Tukey multiple-comparison test in OriginPro 2018. p values < 0.05 were considered statistically significant.

6. Phloxine B-chitosan films as reusable aPDT coatings

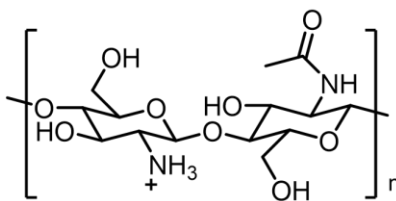
In Chapter 5, the rapid antibacterial activity of PhB-loaded vesicles indicated that internalisation is not a prerequisite for efficient eradication. It was therefore hypothesised that immobilisation of PhB onto a surface could generate $^1\text{O}_2$ at the microbial cell surface, leading to bacterial killing. The final Chapter of this thesis, therefore, shifts the focus from nanoscale delivery systems to macroscopic polymeric coatings, investigating whether surface-bound PhB can be exploited in a more application-oriented format for wound dressings, with longer-term retention of the antimicrobial agent at the dressing surface. Parts of this Chapter are based on a manuscript currently under review and available as a preprint.²³⁴ ii

6.1. Introduction and objectives

In this Chapter, CS films functionalised with PhB are explored as light-activated antimicrobial surfaces, combining the intrinsic properties of CS with localised aPDT. CS is a biodegradable and biocompatible polymer that forms films and is widely used in wound care applications.^{235,236} Its positive surface charge at pH < 6.5 renders it an intrinsically antimicrobial surface; the protonated primary amines (Scheme 9) electrostatically interact with negatively charged microbial membranes, disrupting the membrane integrity, increasing permeability and leading to metabolic dysfunction or cell leakage.^{235–237} These properties have led to extensive use of CS-based materials as haemostatic and antimicrobial wound dressings.

ⁱⁱ Cusick, N; Schönherr, H. Phloxine B-chitosan films as light-activated antibacterial coatings for wound applications. Available at SSRN: <http://dx.doi.org/10.2139/ssrn.6110189>

Author contributions: NC: conceptualisation, methodology, investigation, formal analysis, validation, visualisation, data curation, writing – original draft; HS: supervision, funding acquisition, project administration, resources, writing – review & editing.



Scheme 9. Chemical structure of fully protonated CS at low pH.

Importantly for this work, CS's free primary amines provide an anchor point for the coupling of carboxylic acids to the CS surface.²³⁸ Carbodiimide coupling chemistry has been employed to attach enzyme substrates for bacterial sensing and xanthene-based PSs, such as Rose Bengal.^{239,240} Previous studies have demonstrated that conjugation of halogenated fluorescein derivatives to CS can preserve the $^1\text{O}_2$ -generation ability and confer photodynamic antimicrobial activity, primarily in solution-based or food-preservation contexts.^{240–242}

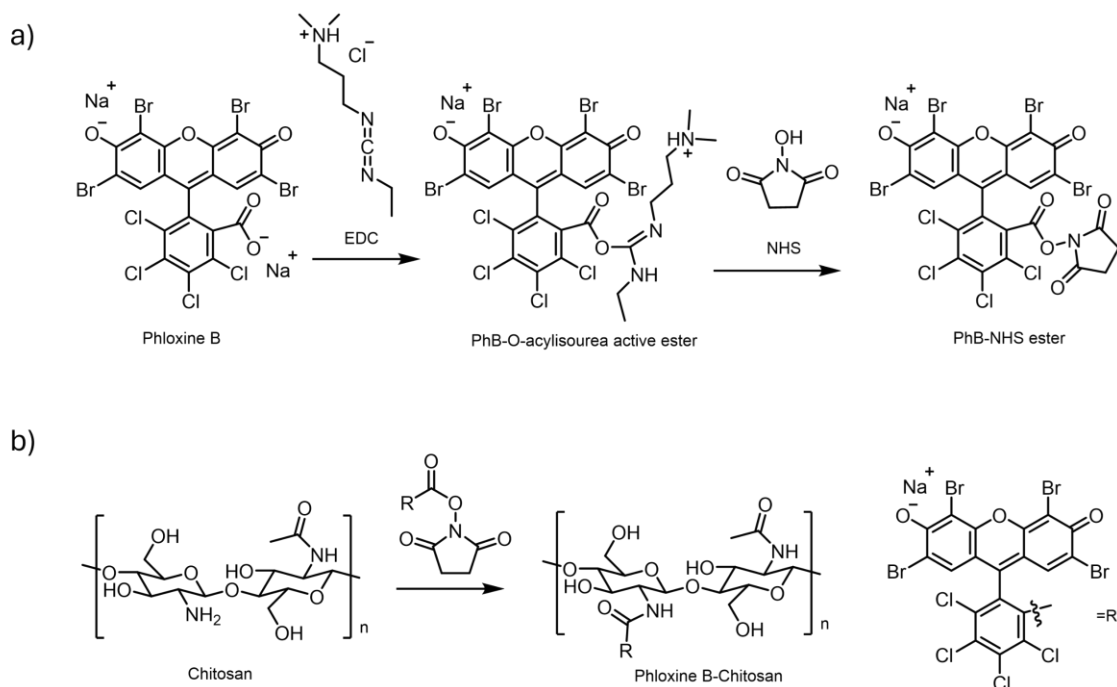
While passive loading of PhB onto surfaces has been reported, these approaches allow free diffusion of PhB away from the target site, reducing long-term efficacy.^{174,243} There has been no investigation into covalently immobilised PhB as an aPDT surface to date. By coupling PhB directly to CS films, this study establishes a non-leaching, contact-active photodynamic coating and evaluates its ability to eradicate bacteria and biofilms.

6.2. CS film formation

CS is soluble in dilute acidic solutions due to the protonation of the free amines, enabling homogeneous solution casting. This method yields uniform films without organic solvent residues, preserving the native chitosan chemistry to facilitate subsequent covalent modification. CS films were formed by casting 1.5 wt% CS in acetic acid into polystyrene Petri dishes and drying overnight. Subsequent neutralisation with a base deprotonates the amines, stabilising the solid film and washing away acetate anions to reduce residual acidity, ensuring mechanical integrity and maximising biocompatibility. The nucleophilicity of the amines is then restored, making them available for coupling.

6.3. Coupling of PhB to CS

EDC/NHS is a zero-length coupling, enabling the formation of amide bonds directly between the primary amines of chitosan and the carboxyl groups of PhB. Importantly, this chemistry is compatible with aqueous conditions, thereby preserving the film's properties. A PhB solution was first exposed to EDC·HCl, which activates the carboxyl group on PhB to form an O-acylisourea intermediate. The addition of NHS stabilises this intermediate as an NHS ester. Upon addition of this PhB-NHS ester solution to the CS film, a nucleophilic attack of the CS primary amines on the PhB-NHS ester leads to the formation of a stable amide bond (Scheme 10).



Scheme 10. a) Activation of Phloxine B with EDC and NHS to form the NHS ester which is then b) coupled to the chitosan backbone.

6.4. Proof of principle

To first determine whether PhB can be coupled to CS, and whether these films would be capable of killing bacteria upon irradiation, small ($\varnothing \sim 1$ cm) CS films were drop-cast and modified with EDC/NHS/PhB. These small PhB-CS films were

washed extensively via Soxhlet extraction for 8 h in 90% EtOH, after which point no PhB diffused from the films. The films were then subjected to a modified Kirby-Bauer (disc diffusion) assay, in which *S. aureus* ($\sim 10^8$ CFU mL⁻¹) was spread using a sterile spreader across an LB agar plate. The films were added on top, then irradiated for a total of 30 min. The agar plates were placed in the incubator (37 °C) overnight to determine the antibacterial effect. It was visible that upon irradiation, there was no bacterial growth between the agar plate and the PhB-CS film, unlike the samples kept in the dark, where bacteria were visible on this surface (Figure 49). Although this method is generally used to determine the antibacterial activity of compounds that have diffused from a surface, it indicates that irradiation provides some anti-*S. aureus* effect and confirms that PhB can be immobilised onto a CS surface.

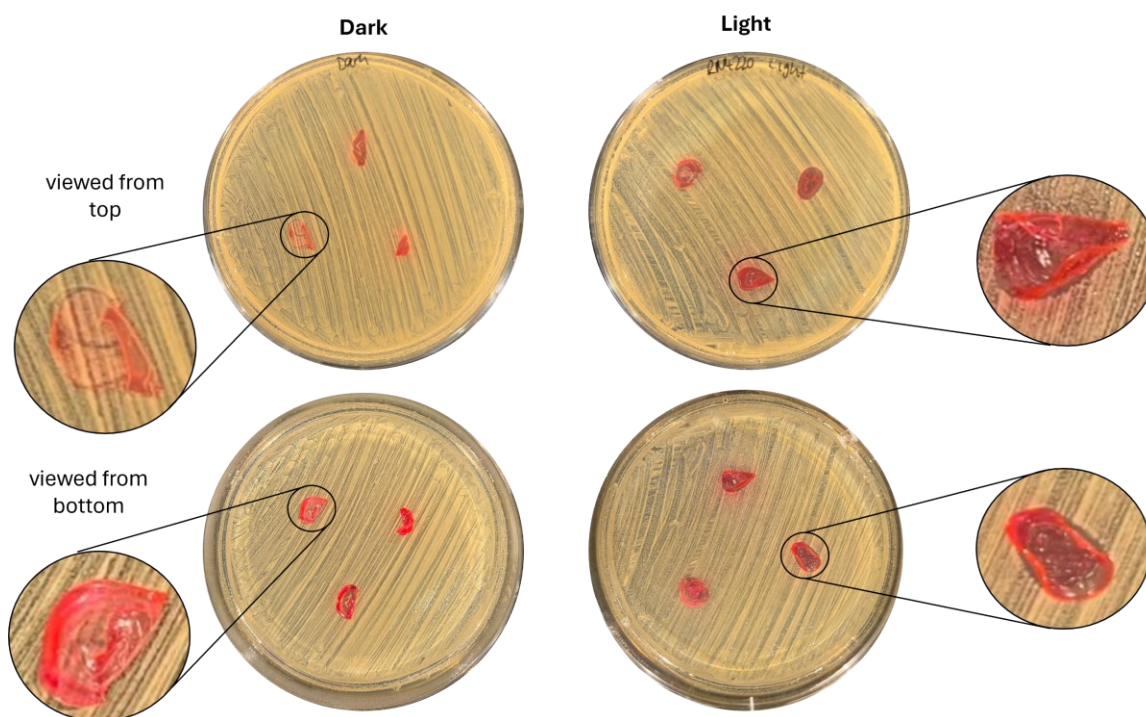


Figure 49. Photographs from the preliminary Kirby-Bauer assay with small samples of PhB-CS. LB agar plates inoculated with *S. aureus* were put in contact with the films, then either kept in the dark for 30 min (Dark) or irradiated from the top and bottom surfaces for 15 min each (Light). After further incubation (18 h at 37 °C), growth was observed visually.

6.5. Upscaling of PhB-CS films

To properly investigate the physicochemical and antimicrobial properties of the PhB-CS films, films with significantly larger surface area were prepared by using larger square Petri dishes (12×12 cm) (Appendix Figure 17). All further results and discussion are regarding films prepared in this manner. As these films were too large to perform Soxhlet extraction, to ensure all free PhB was removed from the films, they were subjected to extensive washing first with Milli-Q water, followed by 20% ethanol until no fluorescence was detected in the washing solution. A fluorescence emission spectrum of the wash was collected to confirm the absence of PhB (Appendix Figure 18). The washed PhB-CS films retained their pink colour (Figure 50). The absence of leached PhB and a permanent colouration are qualitative indicators of successful surface immobilisation of PhB. ATR-FTIR spectroscopy was used to further analyse changes of the functional groups at the surface.

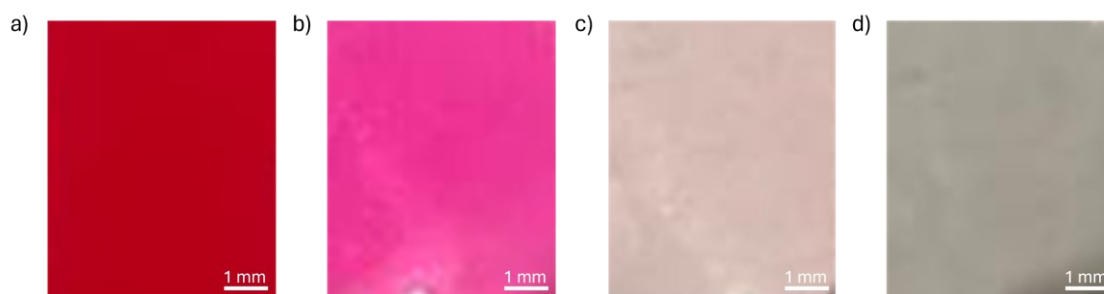


Figure 50. Photographs of a) 1.00 mM PhB-CS, b) 0.10 mM PhB-CS, c) 0.01 mM PhB-CS and d) unmodified CS films.

While the characteristic N-H bending vibrations of the primary amines of chitosan are visible at 1583 cm^{-1} in the unmodified CS film, a clear reduction was observed in the PhB-modified films (Figure 51, Table 4). This provides evidence that the free amine participated in the carbodiimide-mediated coupling with the carboxyl groups of PhB. The coinciding appearance of the prominent amide II (N-H bending) band at $\sim 1552\text{ cm}^{-1}$ and amide I (C=O stretching) at $\sim 1635\text{ cm}^{-1}$ further indicates amide bond

formation. The presence of PhB can be confirmed from the absorption bands observed at $\sim 1458\text{ cm}^{-1}$ and $\sim 1352\text{ cm}^{-1}$, assigned to aromatic C=C stretching and aromatic C-Cl vibrations of PhB. The ATR-FTIR spectrum of PhB and the corresponding band assignments can be seen in Appendix Figure 19 & Appendix Table 2.

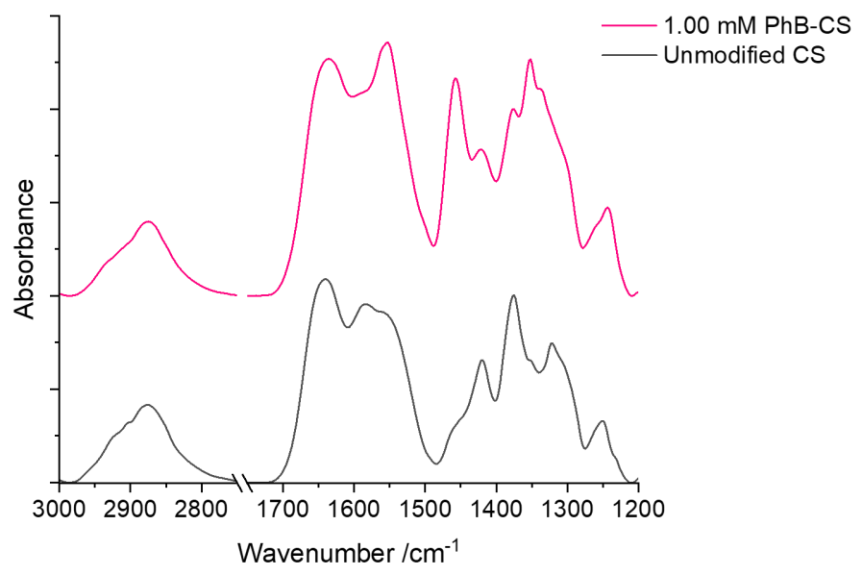


Figure 51. ATR-FTIR spectra of unmodified and 1.00 mM PhB-modified CS films normalised to the C-H bending vibration of CS (1376 cm^{-1}).

Table 4. ATR-FTIR key band assignments for CS, PhB and PhB-CS.

Assignment	Band position (cm^{-1})		
	Unmodified CS	PhB	PhB-CS
$\nu(\text{C-Cl})$ (aromatic)	-	1355	1352
$\nu(\text{C=C})$	-	1458	1458
$\delta(\text{N-H})$ (amide II)	1558	-	1552
$\delta(\text{NH}_2)$	1583	-	-
$\nu(\text{C=O})$ (amide I)	1641	-	1635

The film thickness was investigated by taking a cross-sectional FESEM micrograph of each of the films. Samples were cut and dried overnight in a vacuum oven (25 °C, 5 mbar). The dried films were mounted vertically onto metal nuts using carbon tape and imaged in cross-section by FESEM. Thickness measurements were obtained from the micrographs using ImageJ (Figure 52). All films exhibited thicknesses on the order of $\sim 10\ \mu\text{m}$, typical of such drop-cast CS films.²⁴⁴ The small variations in thickness observed between samples could be attributed to slight differences in the mounting angle during FESEM imaging. Although alternative tools such as micrometres can be used to measure the thickness of such films, FESEM was selected to resolve subtle thickness differences with greater precision. The reduction in thickness observed at higher PhB conjugation levels may indicate dye-polymer interactions that lead to a more compact CS matrix structure. Specifically, hydrogen bonding between the carbonyl and ether oxygens of PhB and the hydroxyl and amine groups of CS can restrict chain rearrangement, resulting in a denser polymer network with reduced free volume upon drying.

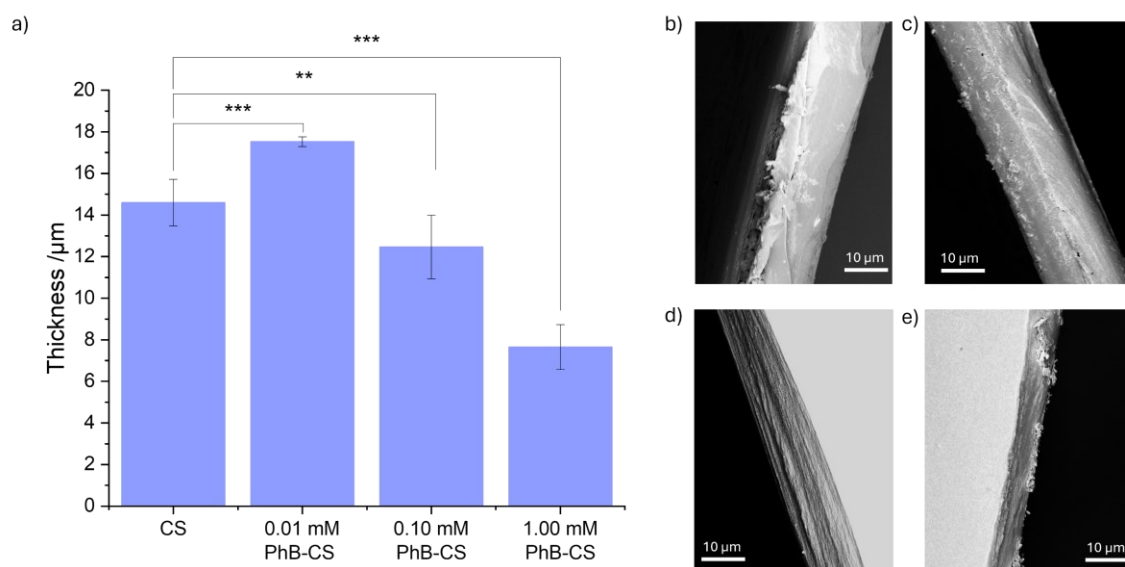


Figure 52. a) Average thickness of unmodified and PhB-modified CS films prepared with increasing PhB concentrations, as determined from FESEM cross-sectional images. Error bars represent standard deviations. Asterisks indicate significant differences compared to unmodified CS (**: $p < 0.01$, ***: $p < 0.001$). b-e) Representative FESEM cross-sectional images of b) unmodified CS, c) 0.01 mM PhB-CS, d) 0.10 mM PhB-CS, e) 1.00 mM PhB-CS. Images were collected with an accelerating voltage of 5 kV, the InLens secondary electron detector and 1000 $\times$ magnification.

The wettability of the films was investigated by measuring the static water contact angle (SWCA). Young's equation describes the contact angle (θ) as a function of the interfacial surface tension (γ) between solid-vapour (SV), solid-liquid (SL) and liquid-vapour (LV) at the three-phase contact line (Equation 11). The SWCA therefore serves as a quantitative indicator of surface hydrophilicity, with lower CAs corresponding to higher surface wettability and greater hydrophilic character, whereas higher CAs indicate reduced water affinity.

$$\gamma_{SV} = \gamma_{SL} + \gamma_{LV} \cos \theta \quad \text{Equation 11}$$

The unmodified CS film displayed a SWCA of $83 \pm 1^\circ$ (Figure 53), which is typical for neutralised CS films with reported SWCAs ~ 60 - 85° , owing to the reduced electrostatic hydration as the amines are largely uncharged. As amines are consumed, the formation of amides lowers γ_{SV} and increases γ_{SL} , decreasing $\cos \theta$, thereby increasing θ . This is evident with the 10° increase in SWCA from unmodified CS to 0.01 mM PhB-CS. A further 33° increase is observed as the concentration is increased to 0.1 mM, indicating further formation of amide bonds. Although free PhB is hydrophilic, when it is covalently immobilised on the CS film, the loss of orientational freedom may lead to the preferential exposure of aromatic regions as the polar groups interact with the bulk CS. This consumption of negatively charged carboxyl groups leads to the loss of ionic hydration of PhB, thereby reducing the free energy of the surface and resulting in a higher contact angle. At higher PhB loading, however, the hydrophilic nature of PhB begins to dominate the surface, promoting favourable interactions with water molecules, causing a decrease in θ . At these high concentrations, the high PhB coverage could also reduce surface roughness due to the denser packing discussed earlier, thereby reducing θ .

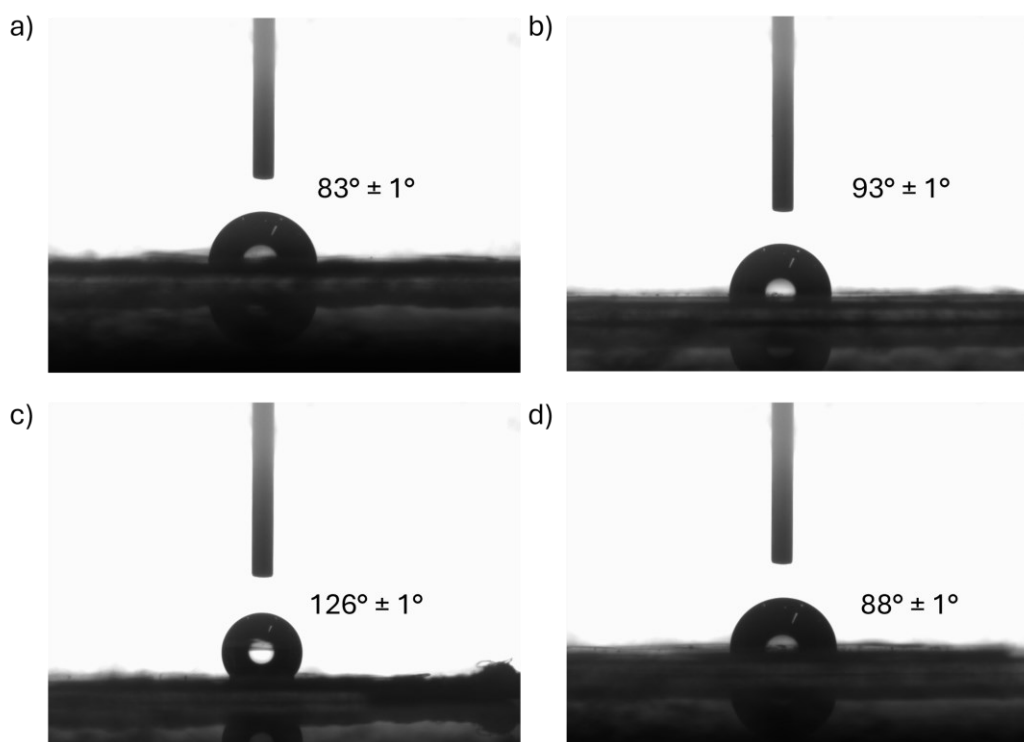


Figure 53. SWCA profiles of a) unmodified CS, b) 0.01 mM PhB-CS, c) 0.10 mM PhB-CS and d) 1.00 mM PhB-CS.

The swelling behaviour of the films was investigated in view of their potential application as wound dressings. While all films could swell to 3-4× their initial mass, all PhB-CS films exhibited a significant reduction in the maximum swelling ratio ($p < 0.001$) compared to the unmodified CS (Figure 54). This observation supports the formation of a denser CS network, as suggested by the thickness studies, which likely restricts water penetration. It could also be due to the increased hydrophobicity as observed in the SWCA measurements (Figure 53). Reported swelling ratios for drop-cast chitosan films vary widely depending on preparation methods. Thin films cast from CS in acetic acid solutions have been reported to reach a 2× increase in mass, whereas films prepared using ionic liquids to improve water solubility can swell up to 21×.^{245,246} Swelling in both cases also reached its plateau after the first measurement point, at 4 hours and 15 min, respectively.^{245,246} Compared to these reports, the moderate swelling observed for

the PhB-CS films is consistent with that observed for films cast from acetic acid solutions.

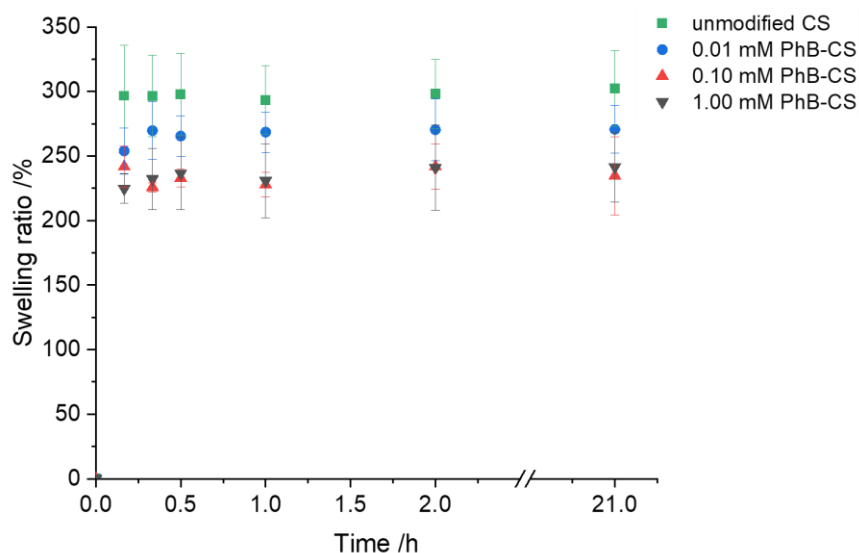


Figure 54. Swelling ratios of unmodified and PhB-modified CS films in PBS (37 °C).

^1H -NMR spectroscopy was attempted to quantify the PhB in the films. However, attempting dissolution of the films in 1% CD_3COOD in D_2O (10 mg mL^{-1}) overnight resulted in the formation of swollen hydrogel particles. After filtering through a $1 \mu\text{m}$ syringe filter, the resulting solution was transparent. The ^1H -NMR spectrum of this solution (Figure 55) exhibited a large singlet peak from HOD at $\sim 4.7 \text{ ppm}$. Traces of ethanol, indicated by a triplet and a quartet at $\sim 1.1 \text{ ppm}$ and $\sim 3.6 \text{ ppm}$, respectively, were observed, suggesting inefficient drying of the films. A further low-intensity multiplet $\sim 2.0 \text{ ppm}$ is likely from the acetic acid protons. Signals from the CS backbone could be assumed at $\sim 3.1 \text{ ppm}$ and $\sim 3.7 \text{ ppm}$, however the concentration of dissolved samples were too low to allow any further analysis using ^1H -NMR spectroscopy. This low intensity of the CS peaks also explains the lack of observable signal from PhB, the aromatic protons of which would be expected in the $6.5\text{--}8.5 \text{ ppm}$ region (Appendix Figure 20). Hence, optical spectroscopy was instead employed.

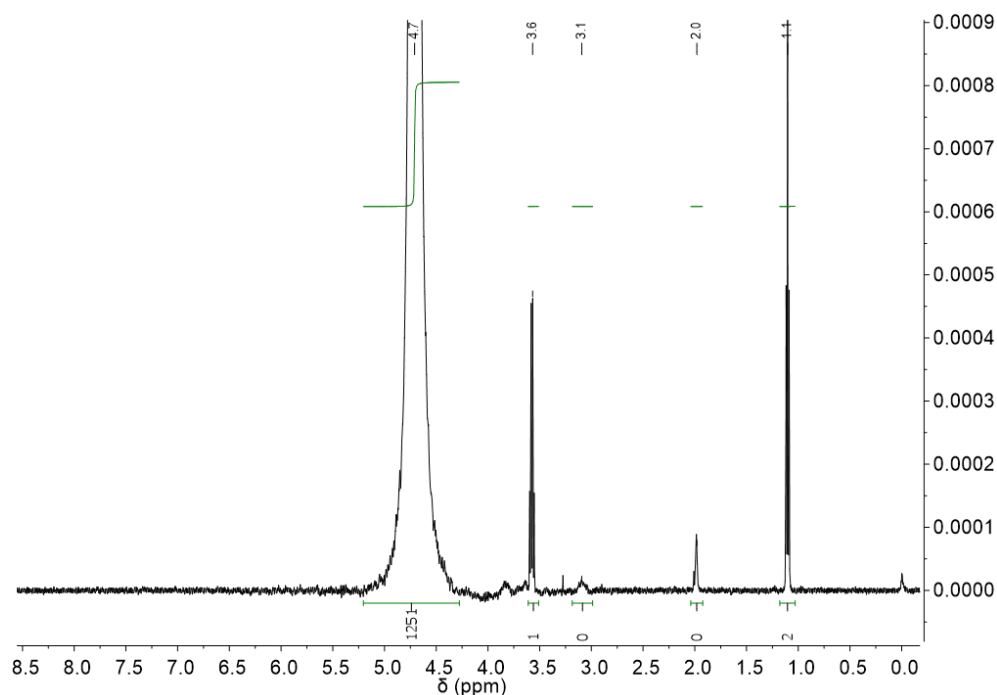


Figure 55. ^1H -NMR spectrum of 1.00 mM PhB-CS in 1% CD_3COOD in D_2O .

6.6. Photophysical behaviour

The normalised absorption and emission spectra of PhB in solution and upon conjugation to CS exhibit clear spectral differences. After immobilisation, a bathochromic shift in the characteristic absorption band of PhB from ~ 539 nm to ~ 555 nm was observed, likely due to the change in local polarity due to interactions with CS (Figure 56a). Small bathochromic shifts have also been observed by Litman et al. upon entrapment of PhB in spin-coated poly(2-hydroxyethyl methacrylate) films, and by Kuryanova et al. upon ionic interaction of the structurally similar Rose Bengal to CS.^{123,247} The effect observed with PhB-CS films is likely larger due to the chemical conjugation of PhB to the polymer. This interaction likely stabilises the excited state of PhB. Upon excitation at 504 nm, the PhB-CS film displayed a broadened emission peak, consistent with restricted molecular motion within the CS network (Figure 56b). The retention of fluorescence in these films also further confirms that PhB remains photoactive after immobilisation, indicating that the coupling reaction does not degrade the chromophore.

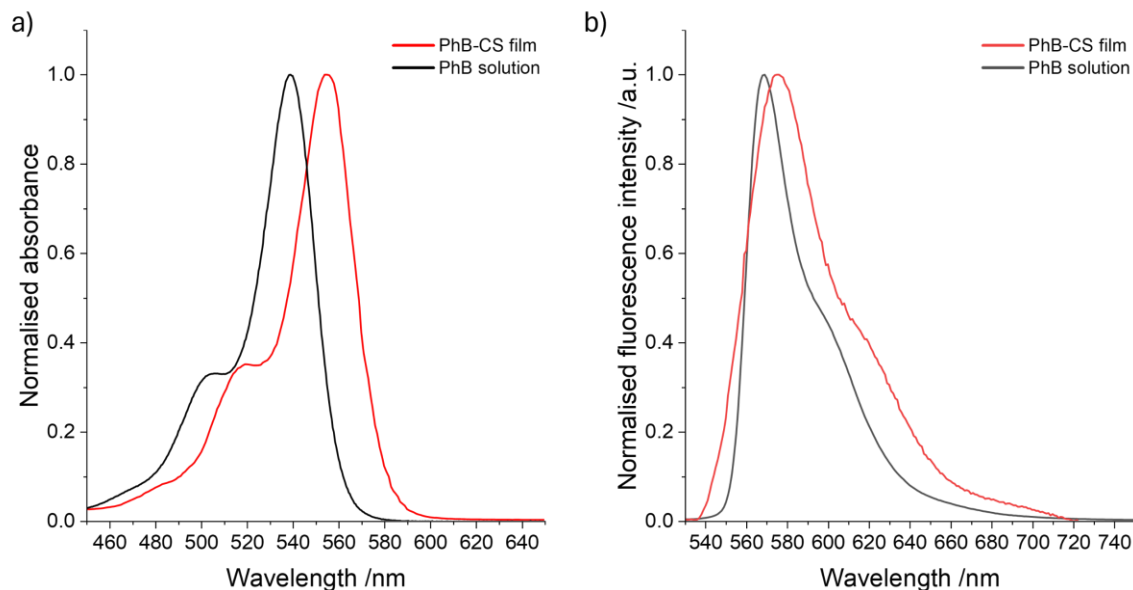


Figure 56. Normalised a) absorbance spectra and b) fluorescence emission spectra ($\lambda_{\text{ex}} = 504 \text{ nm}$) of a PhB solution in Milli-Q water (black) and the dry 0.01 mM PhB-CS film (red).

The absorption and fluorescence emission spectra of all the PhB-loaded films were measured. From the UV-Vis spectra (Figure 57a), the surface concentration (Γ) was estimated using Equation 12, assuming a molar extinction coefficient (ϵ) of $8.3 \times 10^4 \text{ L mol}^{-1} \text{ cm}^{-1}$, as reported in the literature.²⁴⁸ Increasing the PhB concentration from 0.01 mM to 0.1 mM resulted in a $\sim 17\times$ increase in surface concentration from $\sim 1.2 \text{ nmol cm}^{-2}$ to $\sim 19.9 \text{ nmol cm}^{-2}$, from a $10\times$ increase in solution concentration. This indicates that dye modification also involves diffusion into the CS matrix. Using the film thickness to calculate a PhB concentration per film volume, these values become $\sim 0.7 \text{ mM}$ and $\sim 16.6 \text{ mM}$ for the 0.01 mM and 0.10 mM films, respectively. These higher local concentrations are possible because the dye is confined within the CS matrix.

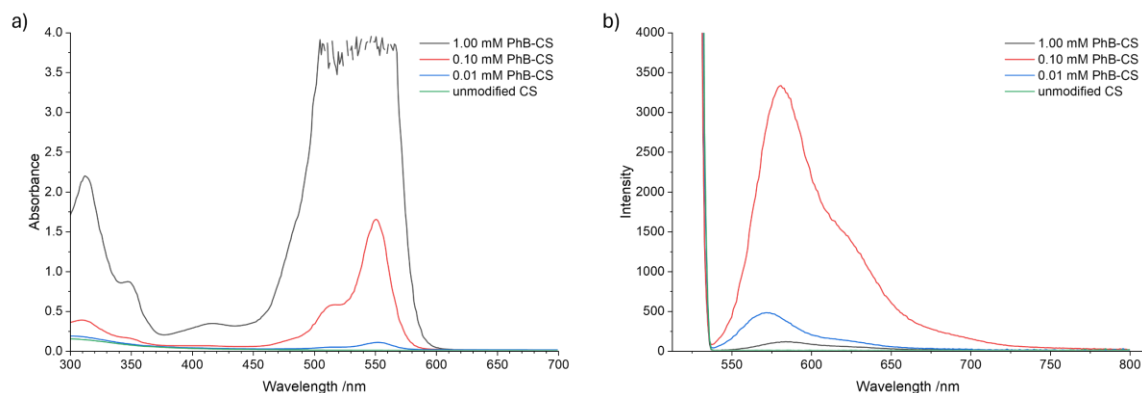


Figure 57. a) Absorbance spectra and b) fluorescence emission spectra ($\lambda_{\text{ex}} = 504 \text{ nm}$) of the CS films with varying PhB concentration from 0.00 mM (green) to 1.00 mM (black).

$$\Gamma = \frac{A}{\varepsilon \times 10^3} \quad \text{Equation 12}$$

Based on the CS mass and assuming an 85% degree of deacetylation, the total stoichiometric density of free amine groups, and therefore the highest number of PhB loading, corresponds to $\sim 11.9 \mu\text{mol cm}^{-2}$, or, assuming a thickness corresponding to the unmodified CS film of $\sim 14 \mu\text{m}$, this corresponds to a maximum theoretical concentration of $\sim 8.5 \text{ M}$. Therefore, even the higher dye loading represents $< 0.2\%$ of total amines have been modified. At the higher PhB concentration of 1 mM , the absorbance of the modified film exceeded the reliable measurement range, precluding quantitative determination of dye loading by UV-Vis spectroscopy. Notably, despite the higher PhB loading and visibly more intense colour, the fluorescence intensity was lower than that of the 0.01 mM film (Figure 57b), consistent with dye aggregation and self-quenching at high local PhB concentrations. The concentration is therefore only known to be $> 16.6 \text{ mM}$.

To evaluate the ability of the PhB-CS films to generate $^1\text{O}_2$, the films were incubated with SOSG, which forms a fluorescent endoperoxide upon reaction with $^1\text{O}_2$.²⁴⁹ By monitoring the fluorescence of the endoperoxide, irradiation-induced ROS-

generation of the films was determined (Figure 58). Control experiments showed a modest increase in background fluorescence from SOSG itself in saline, with unmodified CS producing no additional signal.²⁵⁰ In contrast, PhB-CS films exhibited a pronounced time- and concentration- dependent increase in fluorescence, confirming efficient $^1\text{O}_2$ production. The 1.00 mM PhB-CS film produced the highest SOSG fluorescence, indicating the greatest $^1\text{O}_2$ yield. Notably, all PhB-modified films generated $^1\text{O}_2$ after just 15 min of irradiation, demonstrating that immobilisation preserves photodynamic activity.

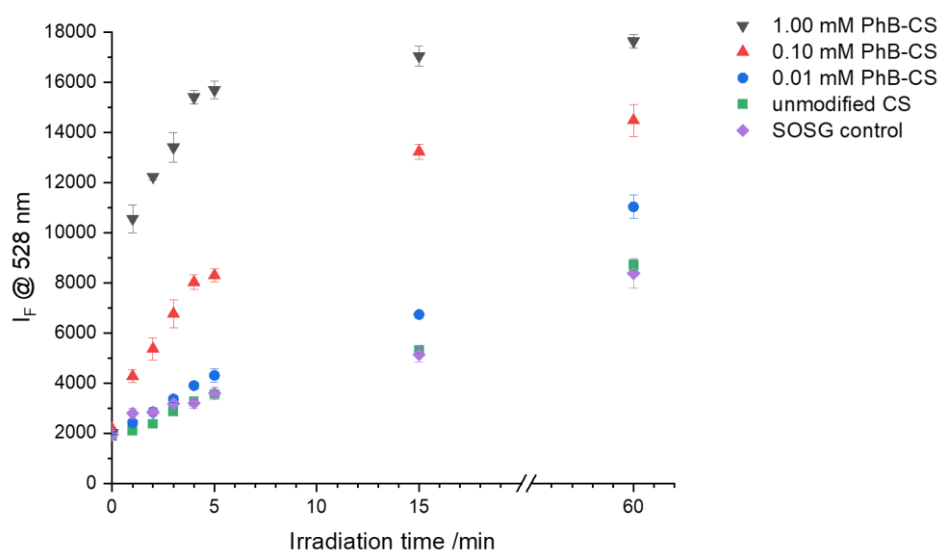


Figure 58. SOSG probe signal with increasing irradiation time ($\lambda = 520$ nm, 4 mW cm^{-2}) of unmodified and PhB-modified CS films.

6.7. Antimicrobial activity

Evaluating the antimicrobial performance of surface-bound materials requires methods designed to ensure bacteria are located at the interface of the material. Especially for non-leaching surfaces, conventional suspension-based assays such as those used in earlier Chapters, are not well-suited due to their inability to determine contact-mediated effects. The Japanese Industrial Standards (JIS) assay JIS Z 2801 (ISO 22196) is designed specifically to quantify the antimicrobial efficacy

of solid surfaces. Here, a defined bacterial inoculum is placed onto the surface and covered to ensure sustained contact. This method is particularly useful to investigate localised ROS generation, but also enables direct comparison with existing antimicrobial surfaces.

Quantification of antimicrobial activity in the JIS assay requires complete recovery of bacteria from the surface. In the case of CS films, swelling as well as possible electrostatic interactions between the cationic polymer and negatively charged bacterial cell envelopes can lead to strong surface adhesion, potentially reducing bacterial recovery. Hence, control experiments were performed to compare bacterial recovery from CS surfaces with that from inert glass substrates. Glass was selected as a non-antimicrobial, smooth reference surface with minimal bacterial retention to assess the efficiency of bacterial recovery. Comparable concentrations of bacteria were recovered from both the CS and glass surfaces, indicating all bacteria are successfully recovered from the films with this method (Figure 59).

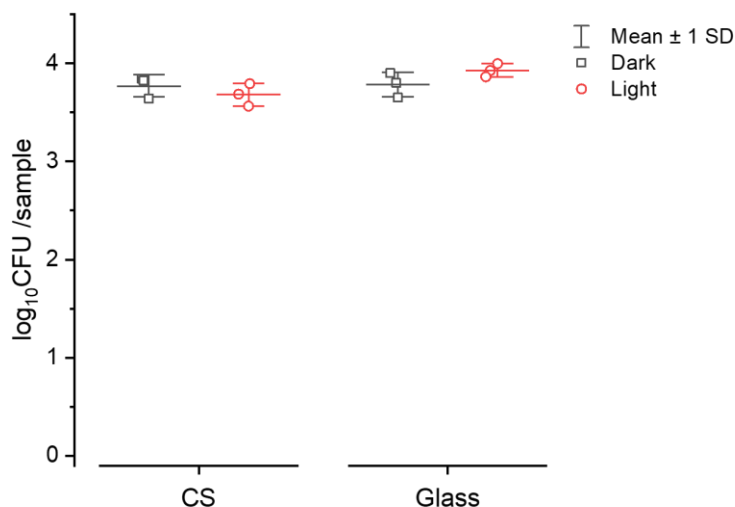


Figure 59. *S. aureus* recovery from non-modified CS films vs glass coverslips.

In all subsequent experiments, unmodified CS films were used as controls. The first experiment with PhB-modified films used the JIS assay standard concentrations, with inocula at low concentrations. A *S. aureus* inoculum of $\sim 7 \times 10^5$ CFU mL⁻¹ ($\sim 2 \times 10^4$ CFU sample⁻¹) was added directly to the film surface, then covered with a

coverslip. Samples were irradiated at 520 nm without any prior incubation from above for either 30 min or 60 min. The distance between the surface and the irradiation source was ~15 mm, owing to the depth of the well.

It was observed that 30 min of irradiation was sufficient for complete eradication of *S. aureus* with the higher concentrated 0.10 mM and 1.00 mM PhB-CS films, while the 0.01 mM film provided only ~99% eradication (Figure 60). The relatively large standard deviation observed for the 0.01 mM PhB-CS film likely reflects inherent variability in ROS-mediated bacterial killing when $^1\text{O}_2$ generation is near the threshold for effective bacterial killing.^{251,252} At this low PhB concentration, minor differences in light exposure, oxygen availability, or bacterial contact with the surface can significantly influence killing efficiency. Moreover, fewer dye molecules coupled to the surface may lead to non-uniform distribution of PhB across the CS film, creating localised variations in photoactivity and contributing to greater variability across biological replicates. A 2× increase in light exposure resulted in complete eradication of planktonic *S. aureus* at this bacterial concentration for all PhB-CS films, further confirming the likelihood that the 0.01 mM film was generating $^1\text{O}_2$ at concentrations close to a critical threshold. Complete consumption of primary amines in the EDC/NHS coupling reaction of PhB with CS is highly unlikely due to diffusion limitations and steric constraints within the solid film, resulting in a net positive surface charge. Especially at lower PhB concentrations, where more of the amines are free, the antimicrobial activity could be an additive effect from the inherent CS activity described previously. It could be hypothesised that there should be an optimal PhB concentration allowing bacterial attachment to the CS surface while maximising the $^1\text{O}_2$ generated in close proximity to the bacteria. This data also confirms that the antimicrobial action is indeed light-triggered; no change in bacterial viability was observed upon incubation of films with bacteria in the dark.

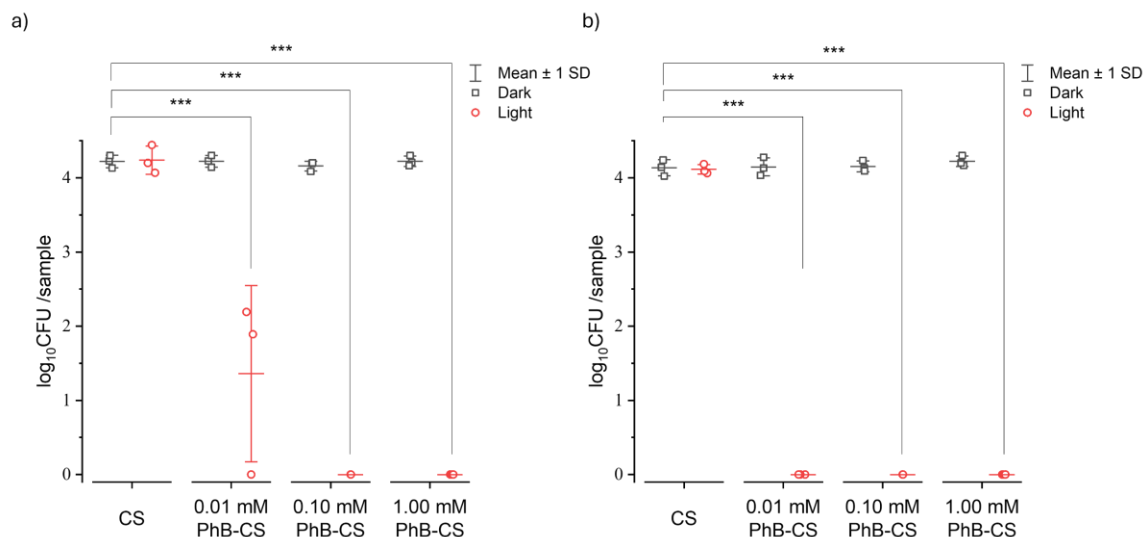


Figure 60. Bacterial counts per sample. Samples were inoculated with *S. aureus* RN4220 at $\sim 7 \times 10^5$ CFU mL⁻¹, then irradiated ($\lambda = 520$ nm, 4 mW cm⁻²) for a) 30 min or b) 60 min. Asterisks represent significant difference from non-irradiated unmodified CS (***: $p < 0.001$).

This low starting inoculum results in lower LRFs despite complete eradication; when accounting for the LOD of 34 CFU mL⁻¹, the complete eradication observed in Figure 60 results in an LRF of ~ 2.5 . To determine whether the PhB-CS films could eradicate *S. aureus* at a higher bacterial load, the inoculum was increased to $\sim 3 \times 10^8$ CFU mL⁻¹ ($\sim 1 \times 10^7$ CFU sample⁻¹). At this higher concentration, complete eradication of *S. aureus* was achieved with all PhB-CS films after 60 min of green light exposure, consistent with the results observed at lower bacteria concentrations (Figure 61), which corresponds to an LRF ~ 5.5 , $>99.999\%$ killing. In contrast, the 0.01 mM PhB-CS film achieved only a 90% reduction in bacterial number, indicating that the lower PhB concentration approaches the threshold for effective $^1\text{O}_2$ -mediated killing, as was observed with a lower inoculum in Figure 60. This therefore suggests that higher PhB loadings or extended light exposure are required to maintain robust photodynamic efficacy under conditions of elevated bacterial burden.

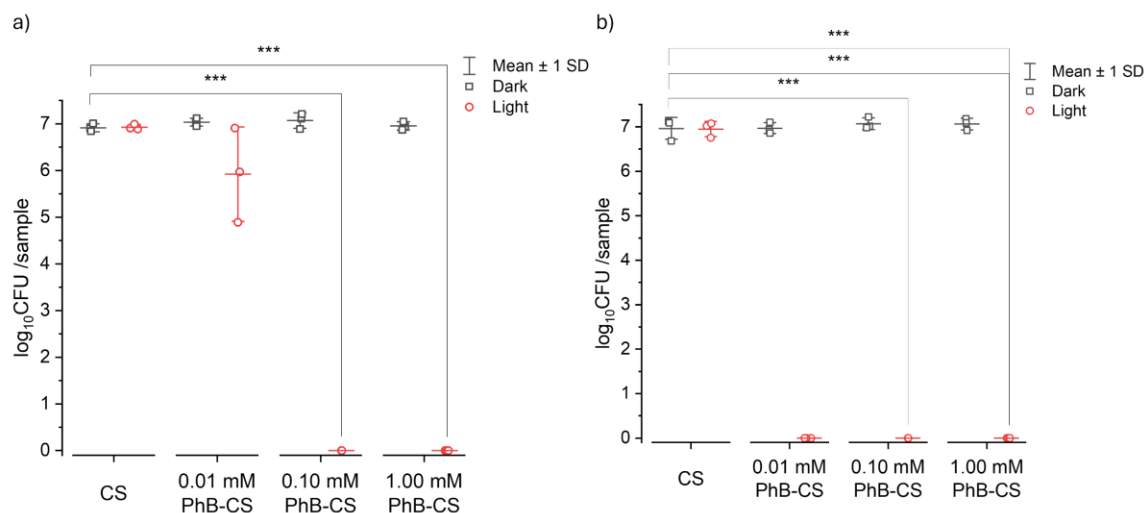


Figure 61. Bacterial counts per sample. Samples were inoculated with *S. aureus* RN4220 at $\sim 3 \times 10^8$ CFU mL⁻¹, then irradiated ($\lambda = 520$ nm, 4 mW cm⁻²) for a) 30 min or b) 60 min. Asterisks represent significant difference from non-irradiated unmodified CS (***: p < 0.001).

To determine whether the antimicrobial effect observed in Chapter 4 against *P. aeruginosa* is also possible with the PhB-CS films, the same experiments were repeated but with *P. aeruginosa*. As expected, it was observed that longer irradiation times were necessary to achieve substantial bacterial reduction (Figure 62). At a lower bacterial concentration, 60 min of green light irradiation resulted in a ~ 3 log₁₀ reduction in *P. aeruginosa* for the 0.10 mM and 1.00 mM PhB-CS films, whereas the 0.01 mM PhB-CS film achieved only $\sim 90\%$ eradication. When the bacterial load was increased, none of the films achieved complete inactivation of *P. aeruginosa*, with only the highest PhB concentration providing any significant reduction in colony counts. These observations are consistent with previous results that indicated higher concentrations of PhB and longer irradiation times are required to eradicate *P. aeruginosa* compared to *S. aureus*, likely due to the protective effect of the outer membrane of Gram-negative bacteria.²⁵³

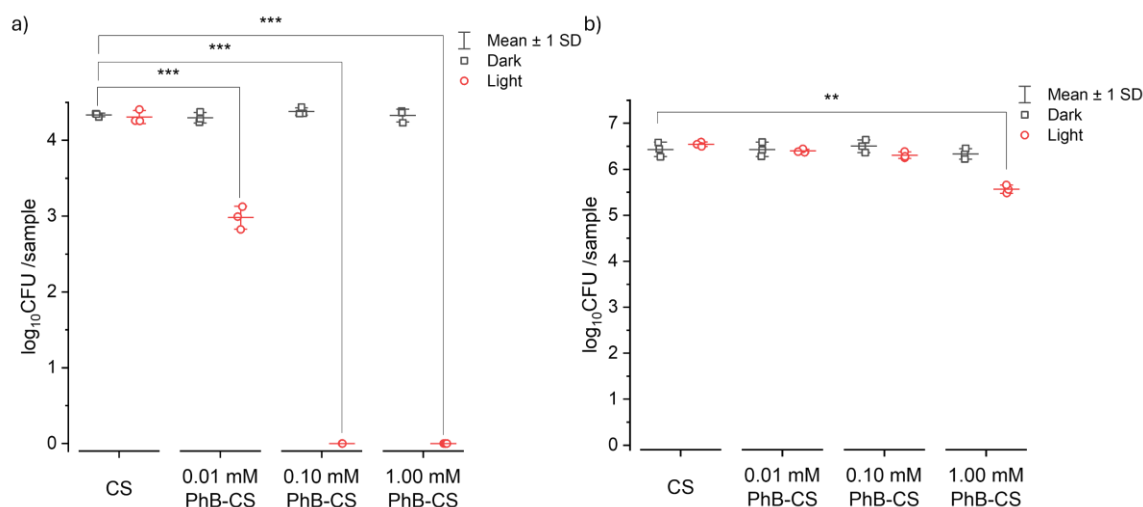


Figure 62. Bacterial counts per sample. Samples were inoculated with *P. aeruginosa* ATCC19660 at a) $\sim 7 \times 10^5$ CFU mL⁻¹ or b) $\sim 3 \times 10^8$ CFU mL⁻¹, then irradiated ($\lambda = 520$ nm, 4 mW cm⁻²) for 60 min. Asterisks represent significant difference from non-irradiated unmodified CS (**: $p < 0.01$, ***: $p < 0.001$).

It was hypothesised that, since the PhB-CS films were shown to be non-leaching, the same film could be used multiple times to kill bacteria. Therefore, after the antibacterial experiments with *S. aureus*, the films were washed extensively with 70% ethanol to sterilise them, then left to soak overnight in Milli-Q water. Spectroscopic analysis of the surrounding solution revealed no detectable fluorescence, confirming that PhB did not leach from the surface under these conditions. The washed films were then placed directly onto sterile agar plates and incubated overnight to determine whether any viable bacteria remained attached to the surface. No colonies were observed after 18 h, confirming the complete removal or inactivation of residual *S. aureus*.

Given the absence of dye leaching and bacteria, the films were subjected to a repeated-use assay to evaluate their durability (Figure 63). Three samples of each film were inoculated with bacteria, irradiated, and the surviving bacteria quantified. After treatment, each film was washed with 70% ethanol and left to dry overnight under a laminar flow hood. The following day, the entire procedure was repeated on

the same films. This approach enabled assessment of the antimicrobial performance of the films across multiple photodynamic cycles and their suitability for potential reuse.

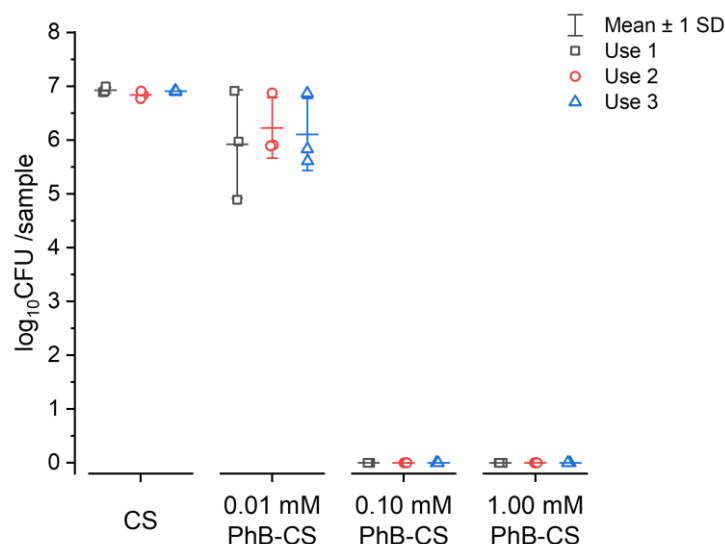


Figure 63. Bacterial counts per sample. Samples were inoculated with *S. aureus* RN4220 at $\sim 3 \times 10^8$ CFU mL⁻¹, then irradiated ($\lambda = 520$ nm, 4 mW cm⁻²) for 30 minutes. Bacteria were recovered and counted (Use 1). Samples were washed in 70% EtOH then soaked overnight in Milli-Q water before the experiment was repeated (Use 2). Washing was repeated and the experiment performed a final time (Use 3).

The 0.01 mM PhB-CS film achieved approximately a 1 log₁₀ reduction in bacterial count, whereas the higher-loaded films consistently produced complete eradication. Notably, no significant decline in antimicrobial performance was observed across repeated treatment cycles for any film type, demonstrating that neither the PS nor the CS matrix undergoes measurable degradation under the irradiation and washing conditions employed. This retention of activity highlights the stability of the films upon irradiation and sterilisation and supports their potential utility in wound care applications, where the ability to perform multiple irradiation sessions on the same dressing without replacing the material could offer substantial clinical benefit.

6.8. Antibiofilm activity

While antimicrobial photodynamic activity is frequently evaluated against planktonic bacteria, the clinical relevance of wound-associated infections is largely governed by biofilm formation at material and tissue interfaces. Biofilms represent a protected, surface-associated mode of microbial growth that significantly reduces susceptibility to antibiotics.

In the context of photodynamic antimicrobial materials, biofilms pose an additional challenge due to the limited penetration depth and short lifetimes of ROS. The PhB-CS films developed in this work are particularly well suited to address this challenge, as they combine electrostatic interactions between the cationic CS matrix and negatively charged biofilm components with localised, light-triggered ROS generation.

Importantly, this study focuses on the eradication of established biofilms rather than the prevention of biofilm formation. Eradication represents a significantly more stringent and clinically relevant challenge. Demonstrating effective bacterial killing under these conditions provides strong evidence that surface-bound photodynamic activity is sufficient to overcome the inherent resilience of biofilms. Biofilms that form in chronic wounds grow at air-liquid and solid-liquid interfaces, with limited nutrient diffusion. The colony biofilm model was therefore selected in this study to replicate important physicochemical features of these wound-associated biofilms, including high local cell density and steep gradients in oxygen and nutrient availability. This model forces bacteria to grow as dense, surface-associated communities, thereby ensuring sustained contact between the biofilm and the CS film (Figure 64). Biofilms were established by inoculating polycarbonate membranes with log-phase bacteria on LB agar, then incubating for 24 h. The resulting so-called “24-h biofilms” attached to the membranes were then transferred to sterile 6-well plates and covered with the CS films. The 1.00 mM PhB-CS film was selected due to its superior $^1\text{O}_2$ generation upon irradiation (Figure 58),

while unmodified CS served as a material control and uncovered biofilms acted as the growth control.

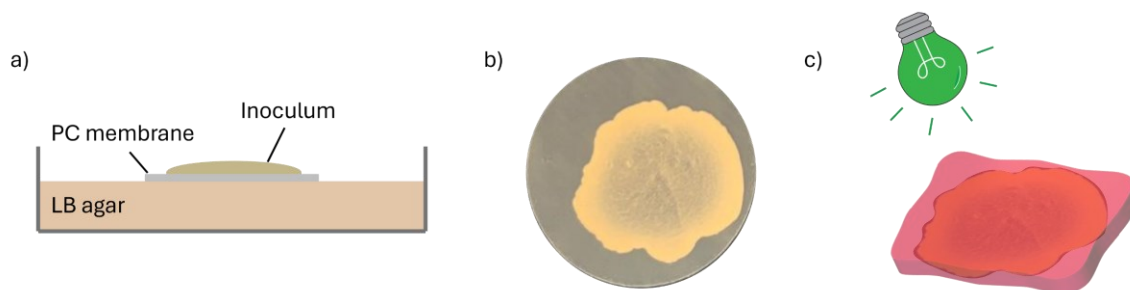


Figure 64. a) Schematic of the colony biofilm model, with a bacterial inoculum grown on a polycarbonate (PC) membrane placed on LB agar, b) top-down photograph of a *S. aureus* RN4220 biofilm, and c) experimental set up for irradiation of PhB-CS films covering biofilms.

Green light irradiation of the growth control and CS film resulted in no change in the viable *S. aureus* count, indicating that neither the light itself nor the unmodified CS surface was toxic to the bacterial cells in a biofilm (Figure 65). Incubation of biofilms beneath the PhB-CS film in the absence of light also resulted in no significant reduction in viable bacteria relative to the growth control, confirming the lack of dark toxicity. In contrast, upon irradiation, a 2 log₁₀ reduction in viable cells was observed. This demonstrates that the immobilised PhB retains sufficient photodynamic activity to significantly disrupt *S. aureus* biofilms, suggesting potential utility for treating persistent *S. aureus* biofilm-associated wound infections.²⁵⁴ Similar reductions in 24 h *S. aureus* biofilms (2-3 LRF) have been observed with PhB-loaded saponite-based nanocomposites, which passively release PhB. This system was limited by the low release (<5% total) of PhB, and will, over time, become less active as the PhB is washed away.^{174,243} These reductions in bacterial count are significantly lower than with planktonic bacteria due to the dense EPS of the biofilm which limits oxygen diffusion. The observed antibiofilm activity in this study therefore reflects the predominantly surface-proximal killing. Moreover, effective aPDT requires sufficient light absorption, but in these experiments, it was necessary to illuminate through the polymer film. This is a

constraint but also representative of the clinical application, in which a wound dressing would have to be illuminated from the exposed surface. It is therefore important to employ thin CS films, and perhaps thinner films would promote more efficient killing by maximising PhB excitation.

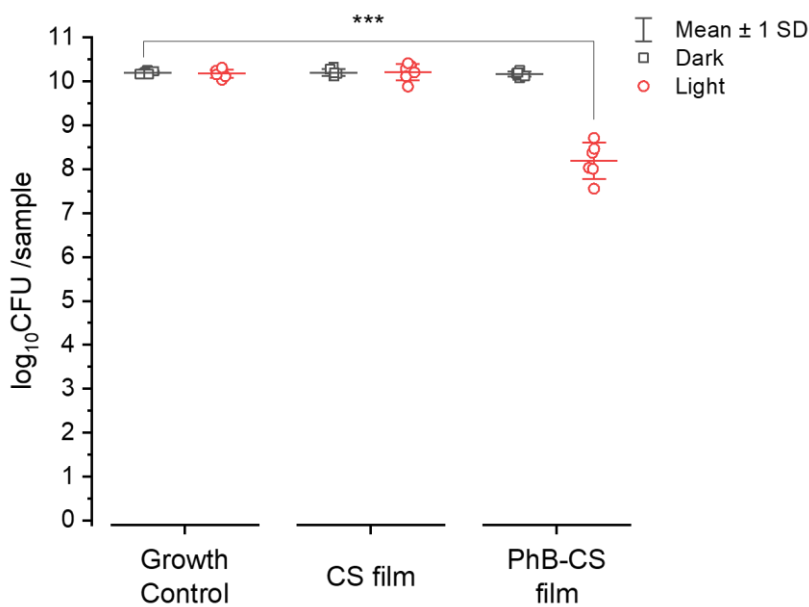


Figure 65. Bacterial counts per sample. 24 h *S. aureus* RN4220 biofilms were grown on polycarbonate membranes, covered with CS films, then irradiated ($\lambda = 520$ nm, 4 mW cm^{-2}) or left in the dark for 60 minutes. Growth control samples were left uncovered. Asterisks represent significant difference from non-irradiated, non-covered growth control (***: $p < 0.001$).

Although larger log reductions in bacterial viability in biofilms have been observed with other immobilised PS systems, such as solutions of Rose Bengal covalently coupled to CS, these systems are not formulated as films, resulting in greater interaction of the PS with the bacteria.²⁴² Since PhB is covalently immobilised onto the CS matrix in this study, the $^1\text{O}_2$ generation is confined to the film–biofilm interface, where it causes membrane oxidation and cell death. Similar modest log reductions have been observed for PhB-modified saponite films.²⁴² The localised mechanism observed with the PhB-CS minimises off-target effects. It is likely that reapplying the film for multiple irradiation cycles could further enhance biofilm

clearance by repeatedly initiating contact-based oxidative killing. However, this could not be evaluated within the constraints of the current experimental setup.

In contrast to the *S. aureus* biofilms, performing the same experiment with *P. aeruginosa* biofilms showed no measurable reduction in viable bacterial cells after irradiation (Figure 66). This outcome is consistent with the reduced susceptibility observed with planktonic *P. aeruginosa* (Figure 62) and aligns with the known protective effect of its outer membrane, resulting in tolerance of Gram-negative biofilms to aPDT.²⁵⁵ It is also hypothesised that a mature *P. aeruginosa* biofilm contains zones with decreased oxygen concentration.²⁵⁶ To eradicate bacteria in such biofilms, higher PS loading and longer irradiation time would be required.

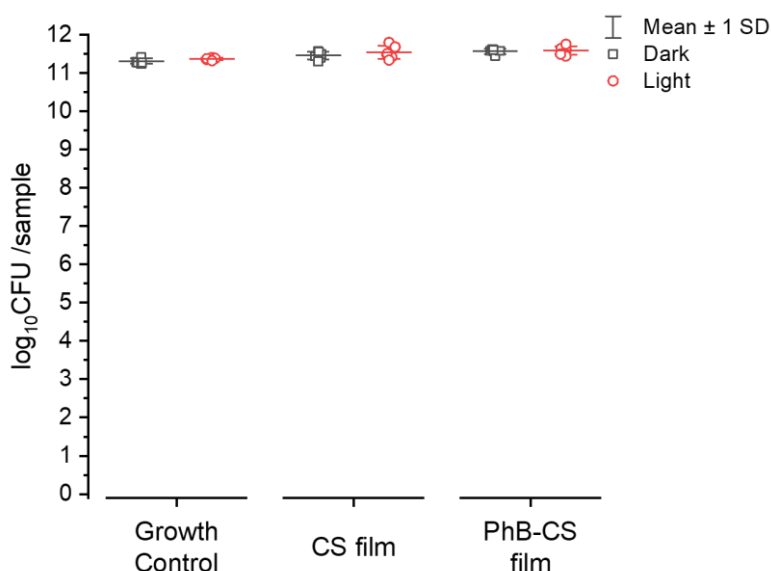


Figure 66. Bacterial counts per sample. 24 h *P. aeruginosa* ATCC19660 biofilms were grown on polycarbonate membranes, covered with CS films, then irradiated ($\lambda = 520$ nm, 4 mW cm^{-2}) or left in the dark for 60 minutes. Growth control samples were left uncovered. Asterisks represent significant difference from non-irradiated, non-covered growth control (***: $p < 0.001$).

6.9. Conclusions and outlook

PhB was successfully immobilised onto a CS matrix to produce stable, non-leaching, photoactive films suitable for aPDT. By covalently anchoring the PhB to the CS surface, a contact-active aPDT platform was established that enables localised, light-triggered bacterial killing without reliance on PS release. This strategy is compatible with existing CS-based wound dressing materials, and on-demand aPDT can be achieved using inexpensive, low-power green LEDs. Importantly, the films retained antimicrobial efficacy over multiple irradiation cycles, enabling multiple treatment applications for the same patient with the same dressing, reducing treatment costs and minimising painful dressing changes.

Conceptually, these photodynamic CS films complement the vesicle-based systems discussed in Chapter 5. While vesicles facilitate the delivery of PS to bacteria in bulk suspensions, the film-based platform exhibits surface-mediated photodynamic killing. The successful eradication of bacteria from the film surface provides further evidence that ROS generation in close proximity to bacterial cells is sufficient to induce cell death, confirming that internalisation of PhB is not required for effective aPDT.

Although significant eradication of *S. aureus* biofilms was achieved, further optimisation is required to address more resistant biofilms. Potential strategies could involve increasing the density of PhB on the surface, introducing spacer molecules such as PEG diamine to enhance accessibility of PhB, or by modifying the surface morphology through patterning or nanofiber formation to increase the effective contact area with the biofilm. In addition, combining surface-bound PhB with membrane-disrupting peptides could provide a promising route to improve efficacy against Gram-negative bacteria, where outer membrane barriers limit photodynamic action.²⁵⁷

6.10. Materials and methods

6.10.1. Materials

Chitosan (CS, medium molecular weight, 190–310 kDa, 75–85 % deacetylated), Phloxine B (PhB, 2,4,5,7-tetrabromo-4,5,6,7-tetrachlorofluorescein disodium salt, dye content $\geq 80\%$), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC·HCl, $\geq 99\%$), *N*-hydroxy succinimide (NHS) were purchased from Sigma-Aldrich, Merck, Germany. Sodium hydroxide (NaOH, $>98.8\%$) was purchased from Carl Roth, Germany.

All solvents were used as purchased: acetic acid (glacial, J. T. Baker, Germany), deuterated water (D_2O , Deutero, Germany), and deuterated acetic acid (CD_3COOD , Sigma-Aldrich, Germany). Milli-Q water was drawn from a Millipore Direct Q8 system with a resistivity of $\geq 18.2\text{ M}\Omega\text{ cm}$ (Millipore Advantage A10 system, Schwalbach, with Millimark Express 40 Filter, Merck, Germany). Acrylic cuvettes and transparent flat-bottom polystyrene 96-well plates were purchased from Sarstedt (Germany), and 12 cm square petri dishes and 96 black half-area μ -clear well plates from Greiner Bio-One (Germany). Glass NMR tubes (Boro 400-5-7) were purchased from Deutero GmbH (Germany). Round glass coverslips (thickness 1, $\varnothing$: 10 mm) were purchased from Carl Roth (Germany). Whatman™ Nuclepore™ Track-Etched membranes (0.2 μm pore size, $\varnothing$: 25 mm) were purchased from VWR, Avantor, Germany.

Lysogeny broth (LB medium, 10 g L⁻¹ tryptone, 5 g L⁻¹ yeast extract, 10 g L⁻¹ NaCl, pH 7.0 $\pm$ 0.2) and LB agar (10 g L⁻¹ tryptone, 5 g L⁻¹ yeast extract, 10 g L⁻¹ NaCl, 15 g L⁻¹ agar-agar, pH 7.0 $\pm$ 0.2) were purchased from Carl Roth GmbH (Germany). Phosphate buffer solution (PBS, pH 7.4) was prepared by dissolving a PBS tablet (Sigma-Aldrich, Merck, Germany) in 200 mL Milli-Q water. Sterile water was purchased from Versylene Fresenius (Bad Homburg, Germany). LB agar, LB medium and PBS were sterilised at 121 °C, 1.2 bar for 15 min in an autoclave

(Systec VB-150, Systec GmbH, Linden, Germany). Simulated thin wound exudate (STWE) was prepared by dissolving NaCl (0.8298%, Sigma-Aldrich, Merck, Germany) and CaCl_2 (0.0368%, AppliChem, Germany) in sterile water. Singlet Oxygen Sensor Green (SOSG) was purchased from Thermo Fisher Scientific (Germany), and Tris buffer (100 mM, pH 7.4) from Millipore (MA, USA).

Pseudomonas aeruginosa lab strain ATCC 19660 (isolated from sepsis in Lima, Peru, and purchased from LGC Standard GmbH (Germany)) and *Staphylococcus aureus* lab strain RN4220 (ATCC 35556; originally derived from NCTC 8325-4 via UV and chemical mutagenesis, purchased from Leibniz-Institute DSMZ GmbH (Germany)) were used in this study. All bacteria were grown in LB medium at 37 °C.

6.10.2. Preparation of CS films

A 1.5 wt% CS solution was prepared in 1% acetic acid and stirred overnight. The solution (35 g) was poured into a 12 cm square petri dish and dried overnight under a flow hood. The resulting films were neutralised with NaOH (20 mL, 0.1 M) for 1 minute on a shaker, then with PBS (20 mL, 2 min).

6.10.3. PhB-CS coupling

PhB solutions (1 mM, 0.1 mM, 0.01 mM) were prepared in PBS. To each stirring PhB solution (50 mL), EDC (0.2875 g) and NHS (0.1725 g) were added, and the mixture was stirred for 1 h. To the neutralised films, 25 mL PhB-EDC-NHS solution was added and left on the shaker overnight. The following day, excess liquid was removed, and the samples were rinsed with excess PBS, then with 20% EtOH, until no dye could be detected in the surrounding media by fluorescence spectroscopy. Emission spectra were recorded using a Varian Cary Eclipse spectrometer (Mulgrave, Victoria, Australia) at 25 °C ($\lambda_{\text{ex}} = 504 \text{ nm}$).

6.10.4. Attenuated total internal reflection-Fourier transform infrared (ATR-FTIR) spectroscopy

ATR-FTIR spectra of the prepared films were recorded on a Tensor 27 Fourier transform infrared spectrometer equipped with a diamond crystal (Vertex Neo R, Bruker Optik GmbH, Germany) in the 600 - 4000 cm^{-1} wavenumber range (non-polarised) with a spectral resolution of 4 cm^{-1} . The background spectra were obtained from measurements against air. Spectra were normalised to the C-H bending vibration of chitosan at 1376 cm^{-1} , which is not present in PhB. Spectra of PhB were measured by first pressing PhB into a KBr pellet, then measuring in ATR mode to avoid contaminating the sample holder.

6.10.5. Static water contact angle (SWCA) measurement

An OCA-15 contact angle microscope (Dataphysics, Filderstadt, Germany) was used in the sessile drop confirmation to measure SWCA. Briefly, a water droplet (2 μL) was dispensed onto the substrate at room temperature. The profile of the drop was used to determine the contact angle. For each film, SWCA was determined 3 $\times$ per sample, across 3 samples, to enable calculation of the mean and standard deviation.

6.10.6. Dry film thickness determination via field emission scanning electron microscopy (FESEM)

Samples were dried overnight in a vacuum oven, then mounted perpendicularly onto a 6-sided metal nut using carbon tape, which was then attached onto an SEM sample holder using carbon tape. To generate a conductive surface, samples were sputtered with gold for 30 s, resulting in a $\sim 5.0 \pm 0.5$ nm gold layer. The sample holder was inserted into the FESEM (Zeiss Ultra 55cv, Zeiss, Oberkochen, Germany). FESEM measurements were collected using the Inlens secondary electron detector, with an operation voltage of 5 kV. Imaging was performed with the assistance of M.Sc. Winny Chelangat. The thickness was determined by

measuring 5 points on each image (3 images per sample) using ImageJ (software version 1.53r). A one-way analysis of variance (ANOVA) with post-hoc Tukey honestly significant difference (HSD) multiple comparison test was performed using OriginPro 2025b to determine the statistical significance of film thickness relative to the unmodified CS film. p values < 0.05 were considered statistically significant.

6.10.7. Swelling behaviour

Swelling behaviour of the prepared films was determined as follows: 2×2 cm square samples were cut and dried overnight in a vacuum oven (25 °C, 5 mbar). The dried hydrogels were weighed (W_d), then placed in 20 mL PBS in closed vials at 37 °C (Heidolph S-biosystems incubator). At regular time intervals, the gels were removed, dabbed lightly with filter paper and weighed (W_s). Each sample was tested in triplicate. Swelling ratio at each time point was calculated according to Equation 13.

$$\text{Swelling ratio (\%)} = \left(\frac{W_s - W_d}{W_d} \right) \times 100 \quad \text{Equation 13}$$

6.10.8. UV-Vis and fluorescence spectroscopy

To confirm the presence of Phloxine B in the modified CS films, the absorption and emission spectra were measured using a Tecan Safire plate reader. Samples were cut into 1×1 cm squares, dried overnight in a vacuum oven (25 °C, 5 mbar), then added to a well of a 12-well polystyrene plate. Absorbance was measured from 400 nm – 700 nm in 1 nm intervals. Fluorescence emission was measured from the top with $\lambda_{\text{ex}} = 504$ nm (bandwidth 10 nm), and emission was measured in 1 nm intervals from 515 nm – 800 nm. For comparison to PhB in solution, a 10 μM solution (in Milli-Q) was measured with the same parameters.

6.10.9. Irradiation experiments

Irradiation of the modified CS films was achieved with a home-built setup described previously.²⁵³ A flat-bottom 96-well polystyrene plate (Sarstedt, Germany) was filled with one green LED bulb per well ($\lambda_{\text{max}} = 520 \text{ nm}$, 4 mW cm^{-2}) and placed directly on top of the well plate to be irradiated. Irradiation was performed in 5-minute intervals until the required irradiation time was reached. As a control, all experiments were performed simultaneously in the dark.

6.10.10. SOSG assay for $^1\text{O}_2$ generation determination

To detect $^1\text{O}_2$, singlet oxygen sensor green (SOSG) was used. A vial (100 μg) of SOSG was dissolved in methanol (33 μL) to prepare a 5 mM stock solution. Aliquots (100 μM in Tris buffer) were stored in the dark (-20°C). To determine $^1\text{O}_2$ generation of the CS films ($1 \times 1 \text{ cm}$), Tris buffer (1800 μL) was added on top of each film in a 12-well plate. SOSG (200 μL) was added, resulting in a final concentration of 10 μM . Aliquots (100 μL) were transferred to a black 96-well plate for measurement of fluorescence emission intensity after regular intervals of light irradiation using a Tecan Safire plate reader ($\lambda_{\text{ex}} = 504 \pm 5 \text{ nm}$, $\lambda_{\text{em}} = 528 \pm 5 \text{ nm}$), then returned to the sample. Each sample was measured in triplicate.

6.10.11. Bacterial culture preparation

LB agar plates inoculated with bacterial glycerol stocks were incubated for 18 h at 37°C . A single colony was taken from this plate and used to inoculate LB medium (5 mL). This tube was incubated for 18 h at 37°C while shaking at 200 rpm. Bacterial pellets were isolated by centrifugation ($5000 \times g$, 5 min, 4°C) and the supernatant was exchanged with STWE. The optical density at 600 nm (OD_{600}) was adjusted in STWE to 0.6 (*P. aeruginosa*) or 1.2 (*S. aureus*), corresponding to $\sim 10^8 \text{ CFU mL}^{-1}$.

6.10.12. Modified Kirby-Bauer assay

Prior to any antibacterial assays, samples were immersed in 70% ethanol for 30 min, then in sterile water for 30 min, and then dried overnight in the sterile hood.

A circular LB agar plate was inoculated with *S. aureus* RN4220 ($\sim 10^8$ CFU mL⁻¹) by placing a sterile swab into the inoculum, then streaking the plate with the swab to ensure complete coverage. PhB-CS films ($\varnothing \sim 1$ cm) were placed on top of the agar plate (3× per condition). Plates were either kept in the dark for 30 min or irradiated for 15 min from the top surface and 15 min from the bottom surface. After incubation for 18 h at 37 °C, photographs were taken to observe the bacterial growth.

6.10.13. Surface bactericidal activity assay with planktonic bacteria

A modified JIS assay adapted from Schmitz et al. was used to evaluate the bactericidal activity of the PhB-CS films.²⁵⁸ Sterile samples were transferred to a sterile 12-well flat-bottom polystyrene plate. To determine activity against planktonic bacteria, bacterial suspension (30 μ L) was added on top of films (in triplicate) and covered with a glass coverslip ($\varnothing = 10$ mm). Samples were irradiated directly for 30 or 60 min by placing the light source on top of the well plate. Non-irradiated controls were kept in the dark for the same time duration. The maximum irradiation applied in this study provided an irradiance of 4 mW cm⁻² for 60 min, corresponding to a total fluence of 14.4 J cm⁻². Assuming complete energy absorption and no heat dissipation (worst-case scenario), the theoretical maximum temperature increase in hydrated tissue ($c = 3.4$ kJ kg⁻¹ °C⁻¹) would be approximately 3–4 °C.²⁵⁹ In practice, heat dissipation through conduction and perfusion, as well as partial optical reflection and scattering, further limit temperature elevation. Therefore, the applied irradiation conditions are not expected to induce thermally-mediated tissue damage.²⁶⁰

To determine the number of viable bacteria remaining, PBS (2 mL) was added to each well and mixed thoroughly. Well plates were sonicated (Bandelin Sonorex Digiplus, 35 kHz bath sonicator, nominal power 160 W) for 10 min at 25 °C, then serially diluted (10×) in PBS, and 3 × 10 μ L was plated for each dilution step on LB

agar plates, resulting in an LOD of 34 CFU mL⁻¹. Bacterial colonies were counted after incubation for 16 h at 37 °C and used to determine the log₁₀ CFU mL⁻¹. To confirm all bacteria were recovered from the CS surfaces, a control experiment with glass coverslips (ø = 10 mm) was first performed. All experiments were performed in biological triplicate. A one-way ANOVA with post-hoc Tukey HSD multiple comparison test was performed using OriginPro 2025b to determine statistical significance. *p* values < 0.05 were considered statistically significant.

6.10.14. Antibacterial assay with *S. aureus* and *P. aeruginosa* biofilms

Polycarbonate membranes were sterilised under a UV transilluminator (UVP m-20V, λ = 302 nm) for 10 min on each side prior to use. Sterilisation of the membranes was confirmed by incubating them in LB (5 mL) for 18 hours at 37 °C, with no growth confirming sufficient sterilisation. The procedure for creating a biofilm was adapted from Merritt et al.²⁶¹ Briefly, membranes were then transferred with sterile tweezers to square LB agar plates. STWE (30 µL) was added to the membrane and left to dry (~15 min) in a sterile laminar flow hood. Bacteria suspension (~10⁶ CFU mL⁻¹ in STWE, 50 µL) was added on top of the membrane. Afterwards, membranes were incubated for 24 hours to allow biofilm growth, then transferred to sterile 6-well plates. Dry CS films were placed directly on top of the biofilms. Samples were irradiated from the top (60 min) or kept in the dark for the same time. After irradiation, PBS (2 mL) was added to each well and mixed thoroughly. Well plates were sonicated (10 min, 25 °C, 35 kHz, 160 W nominal power) then the contents of each well serially diluted (10×) in PBS, and 3 × 10 µL was plated for each dilution step on LB agar plates, resulting in a LOD of 34 CFU mL⁻¹. Bacterial colonies were counted after incubation for 16 h at 37 °C and used to determine the log₁₀ CFU mL⁻¹. All experiments were performed in biological sextuplicate.

7. Summary and outlook

This Thesis addressed the development of polymer-based photodynamic antimicrobial systems for potential application in future advanced wound dressings, with a particular focus on the FDA-approved xanthene dye Phloxine B (PhB) as a photosensitiser (PS) for antimicrobial photodynamic therapy (aPDT). This work aimed to advance the incorporation of small-molecule PSs into functional polymeric platforms capable of localised and effective bacterial inactivation.

In Chapter 4, the photophysical properties and aPDT activity of PhB were systematically investigated. Absorption and emission characteristics, singlet oxygen generation, and photostability were evaluated, providing insight into molecular PhB. The antimicrobial efficacy of PhB was demonstrated against clinically relevant Gram-positive *Staphylococcus aureus* and Gram-negative *Pseudomonas aeruginosa*, two major pathogens associated with wound infections. Under defined irradiation conditions ($\lambda = 520 \text{ nm}$, 4 mW cm^{-2}), PhB achieved bacterial reductions of up to $7 \log_{10}$, confirming its suitability for aPDT applications. Efficacy was shown to depend on PhB concentration, irradiation time, bacterial species and the nature of the surrounding medium.

Building on these findings, Chapter 5 focused on the synthesis and characterisation of amphiphilic poly(ethylene glycol)-*block*-polyester (PEG-*b*-polyester) copolymers and their self-assembly into nanoscale architectures for the encapsulation of PhB. By varying the hydrophobic block length, polymer assemblies with diameters ranging from approximately 50–200 nm were obtained. Due to its water solubility, PhB was preferentially encapsulated within polymeric vesicles of $\sim 200 \text{ nm}$ in diameter, yielding vesicle suspensions with dye concentrations exceeding $30 \mu\text{M}$. These PhB-loaded vesicles retained strong photodynamic activity, reducing the burden of both *S. aureus* and *P. aeruginosa* below the limit of detection after 15 min of green light irradiation. However, the short timescales required for bacterial

inactivation suggested limited cellular internalisation of PhB, indicating that extracellular or surface-mediated photodynamic mechanisms dominated.

Motivated by these observations, Chapter 6 explored the immobilisation of PhB within chitosan (CS) films to create photoactive antimicrobial surfaces. The resulting PhB–CS films enabled contact-based bacterial killing upon light activation and demonstrated effective inactivation of both planktonic and biofilm-associated bacteria, with reductions of up to 6 log₁₀ and 2 log₁₀, respectively. The successful antimicrobial activity of the immobilised PhB confirmed that bacterial internalisation was not required. A significant advantage was the ability to reuse the films, allowing multiple irradiation cycles. These findings highlight the potential of naturally derived polymers such as CS, which are already employed in wound dressing materials, for the development of sustainable, light-activated, antimicrobial advanced wound dressings.

Despite the promising results presented in this Thesis, further work is certainly required to advance these systems toward clinical translation. Future studies should investigate the long-term photostability, reusability, and mechanical robustness of PhB-containing polymeric materials under physiologically relevant conditions. Investigation of minimum irradiation times and dosage to improve patient compliance is necessary. In addition, evaluating cytocompatibility with relevant mammalian cell types and assessing performance in more complex wound-mimicking models will be essential. Determination of *in vivo* antimicrobial efficacy and potential off-target effects will be necessary to support future approval. Upon demonstration of safety and performance, progression to first-in-human Phase I clinical trials focusing on safety, tolerability, and preliminary efficacy would be the next critical step toward clinical translation.

Overall, this Thesis provides a foundation for the rational design of PhB-based photodynamic materials and contributes to the broader development of alternative antimicrobial strategies amid increasing antimicrobial resistance.

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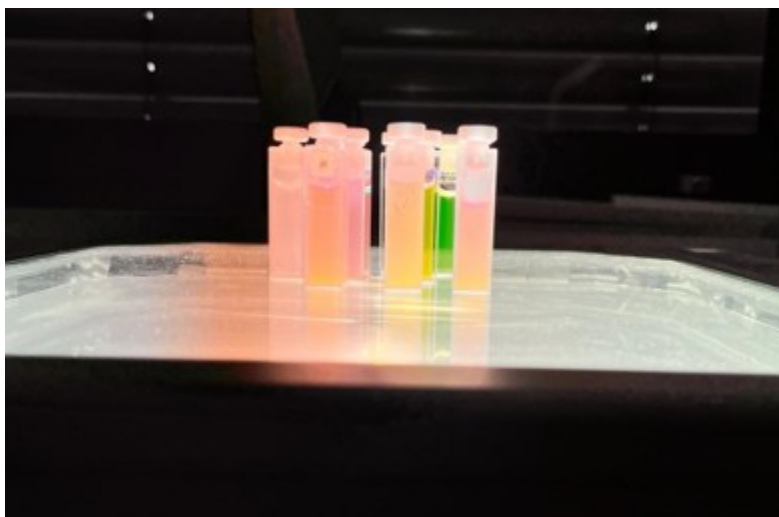
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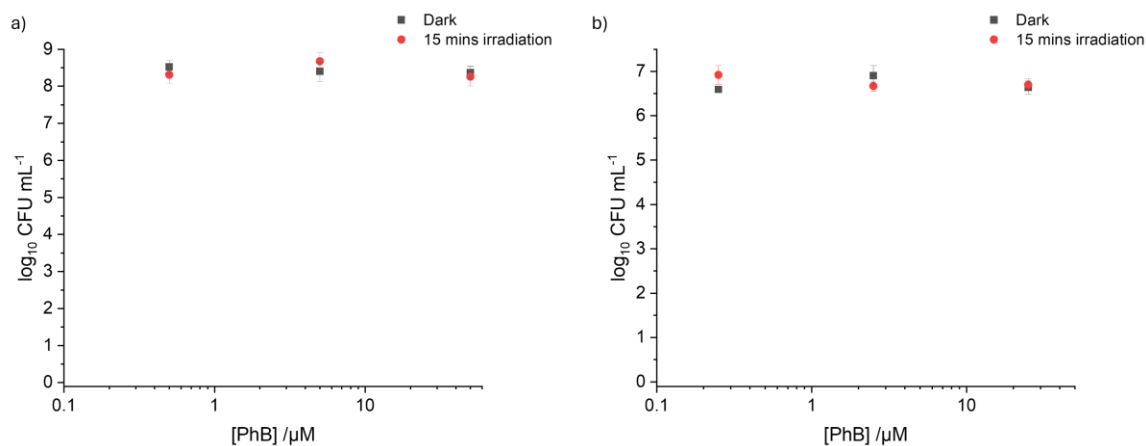
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9. Appendices

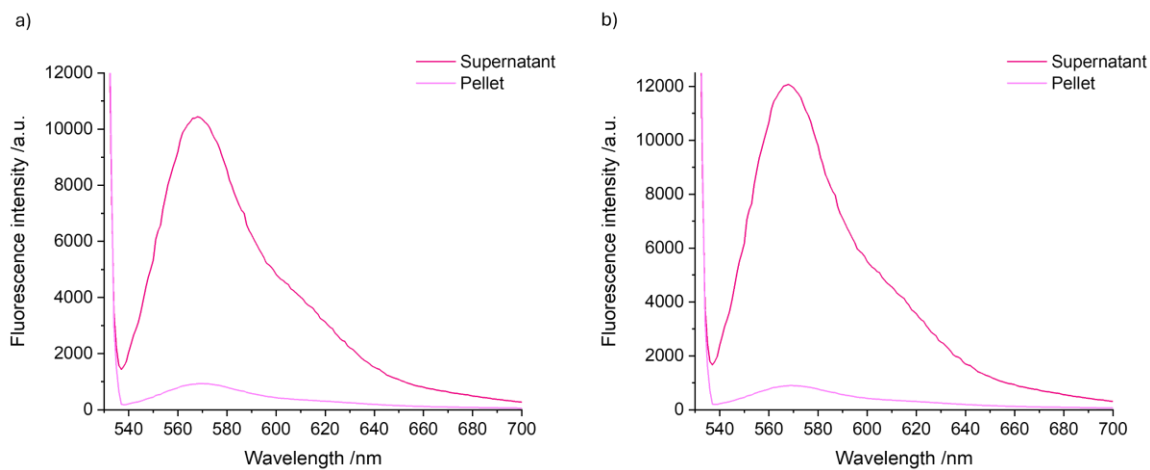
9.1. Chapter 4 appendix



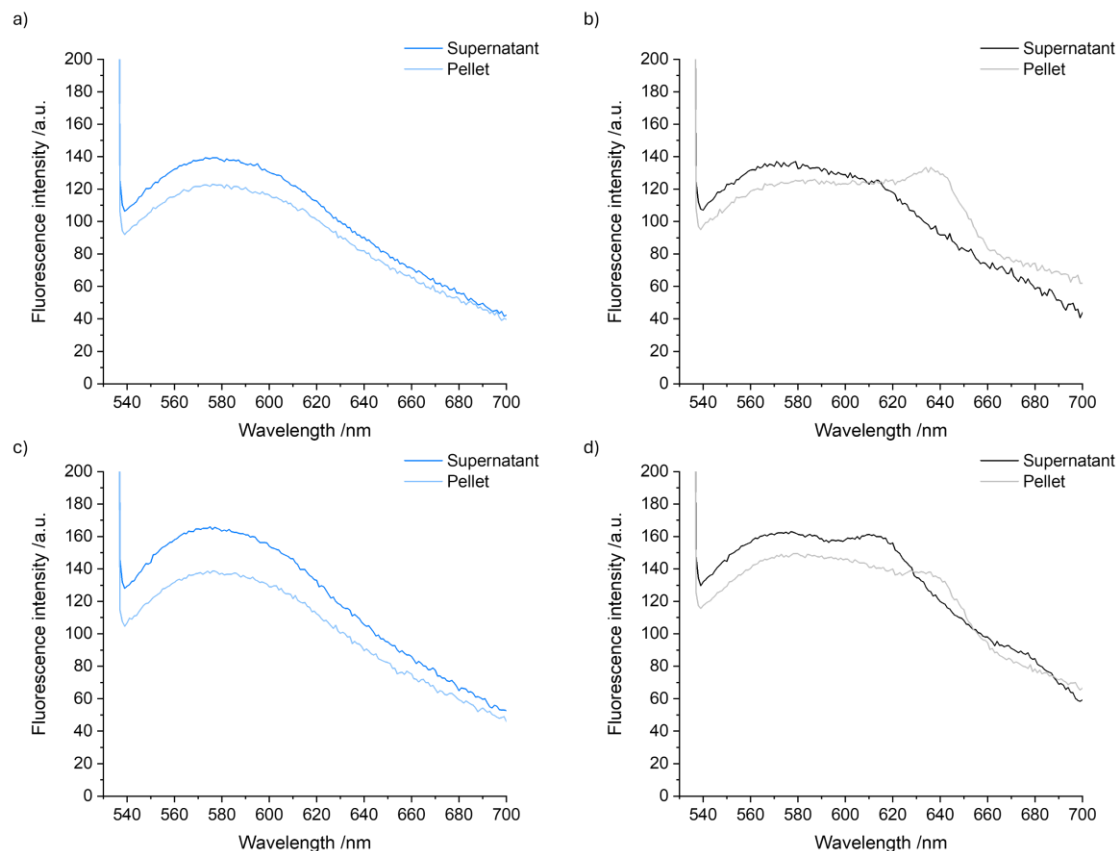
Appendix Figure 1. Irradiation of dye solutions using an overhead projector for the determination of photostability.



Appendix Figure 2. Effect of PhB concentration (logarithmic scale) on a) *S. aureus* RN4220 and b) *P. aeruginosa* ATCC19660 in simulated wound fluid. CFU counts following treatment with free PhB under different irradiation conditions (Dark or 15 min irradiation ($\lambda = 520 \text{ nm}$, 4 mW cm^{-2})). Experiments were performed in technical triplicate.

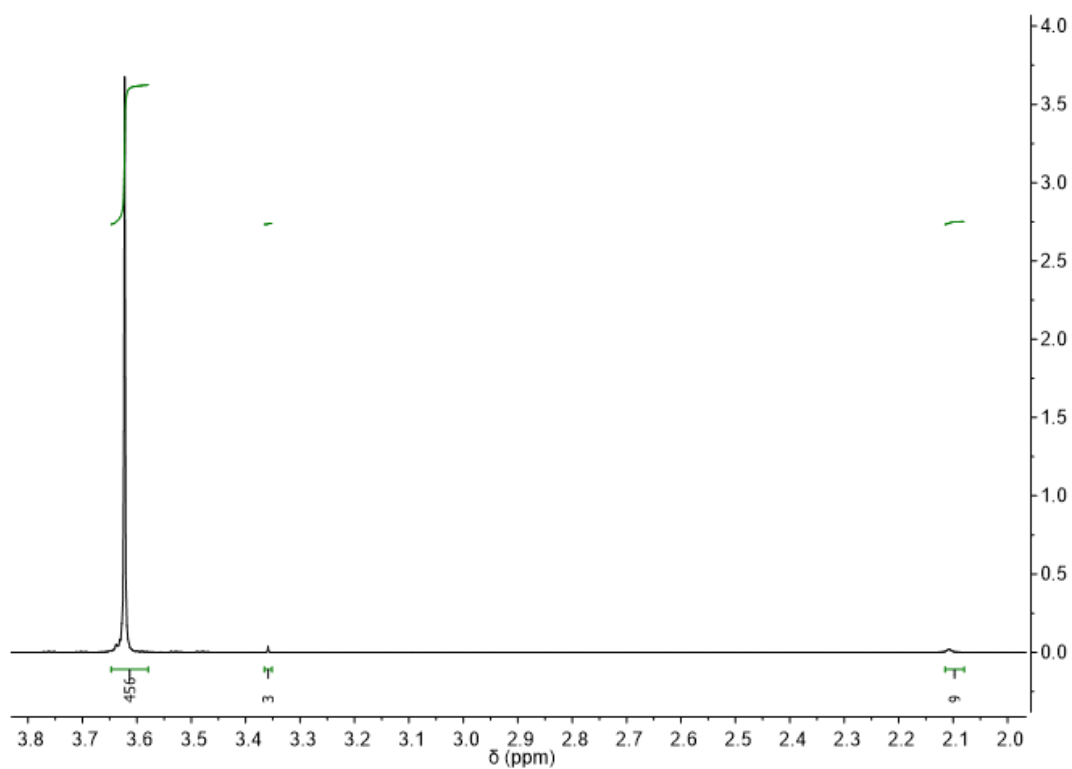


Appendix Figure 3. Control experiments of PhB emission in partitioning experiments. PhB (5 μ M) was incubated in Eppendorf tubes for a) 15 min or b) 16 h, followed by centrifugation. Fluorescence emission spectra were recorded for the supernatants and for the resuspended “pellet” of leftover PhB in the Eppendorf tube (λ_{ex} = 504 nm).

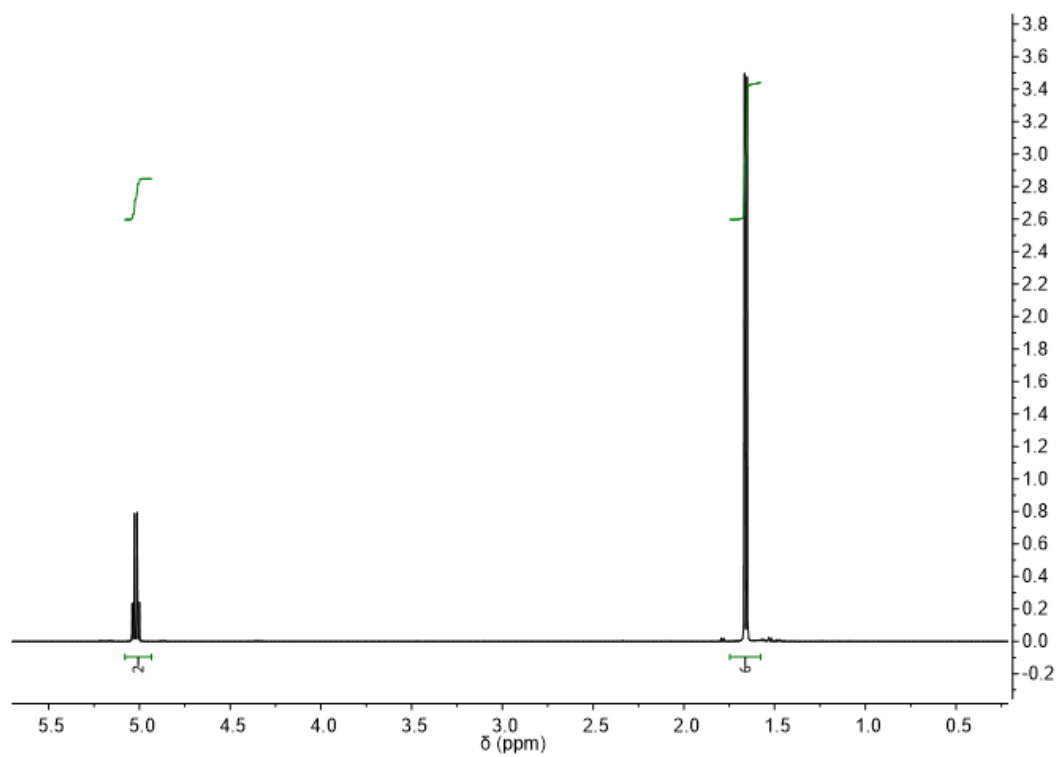


Appendix Figure 4. Assessment of inherent fluorescence of a) *S. aureus* and b) *P. aeruginosa*. PhB association with bacterial cells by fluorescence partitioning. Bacteria ($\sim 10^8$ CFU mL⁻¹) were incubated with Milli-Q for a-b) 15 min or c-d) 16 h, followed by centrifugation to separate bacterial pellets and supernatants. Fluorescence emission spectra were recorded for the supernatants and for resuspended bacterial pellets ($\lambda_{ex} = 504$ nm).

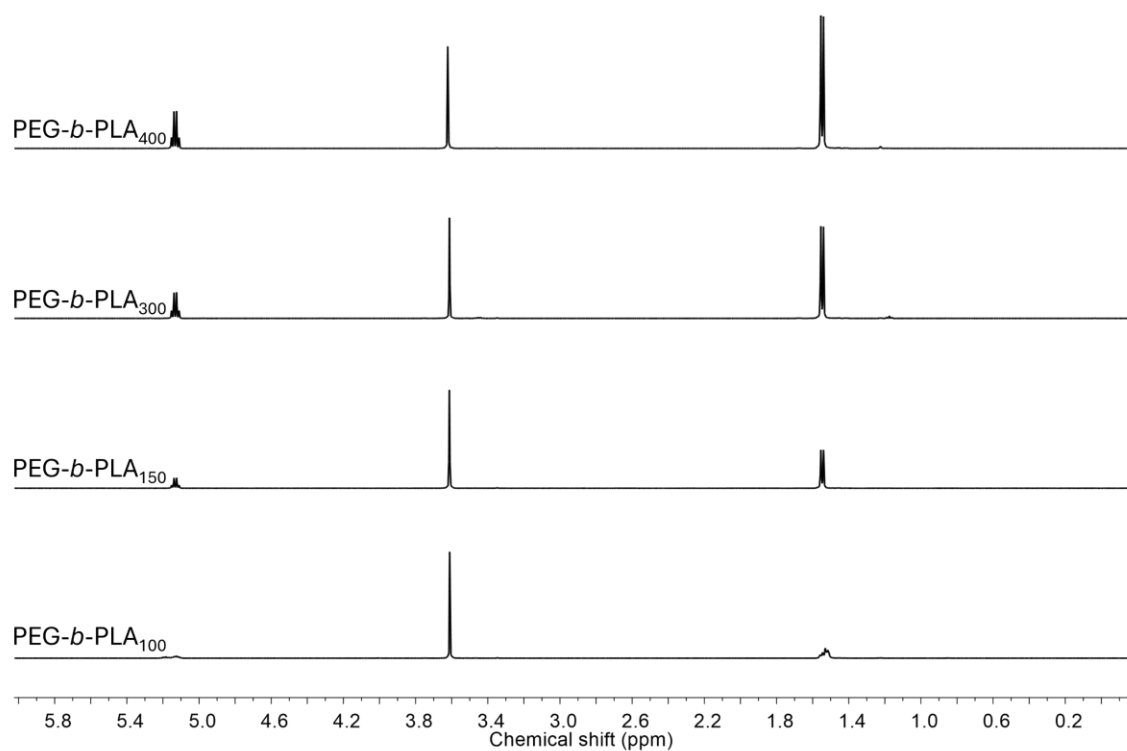
9.2. Chapter 5 appendix



Appendix Figure 5. ^1H -NMR spectrum of mPEG in CDCl_3 .



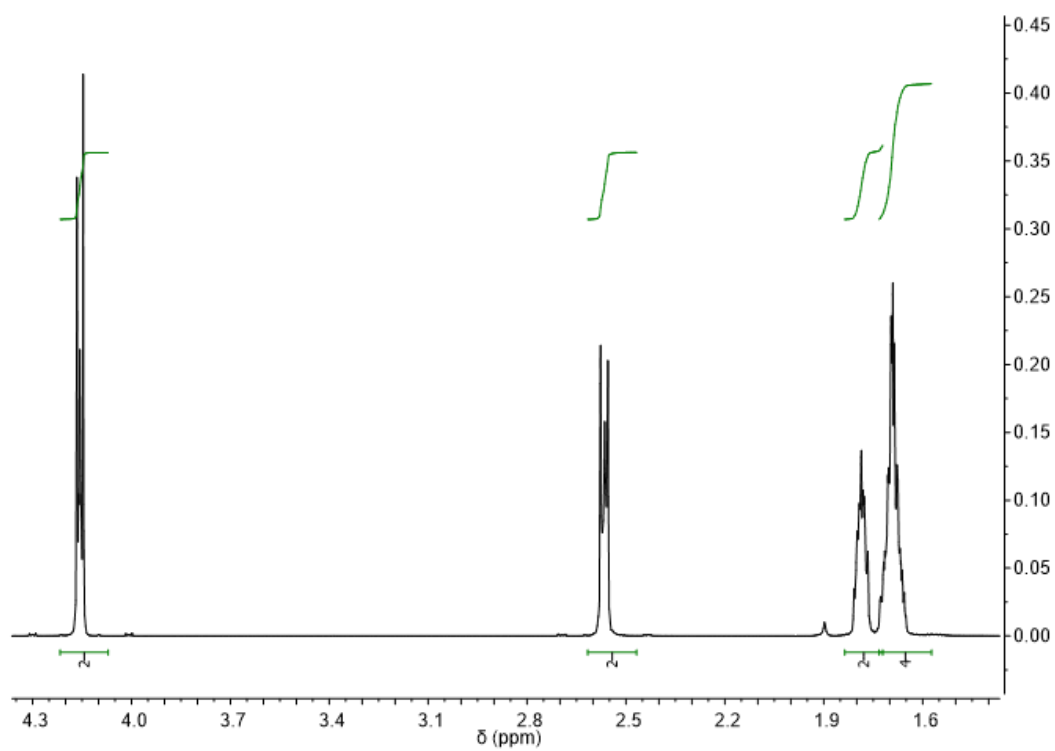
Appendix Figure 6. ^1H -NMR spectrum of L-lactide in CDCl_3 .



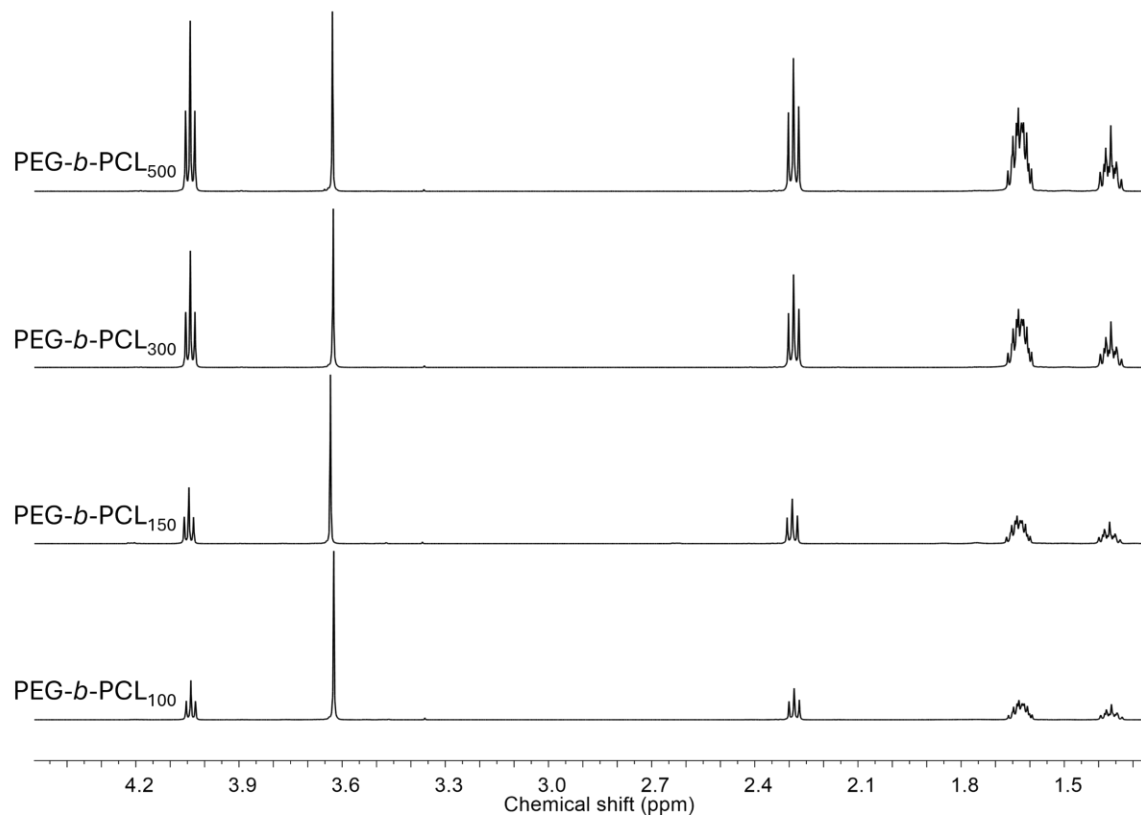
Appendix Figure 7. ^1H -NMR spectra of $\text{PEG}_{114}\text{-b-PLA}_x$ in CDCl_3 , with $x = 100, 150, 300$ and 400 .

Appendix Table 1. ATR-FTIR key band assignments for mPEG, PLA, PCL, PEG-b-PLA and PEG-b-PCL .

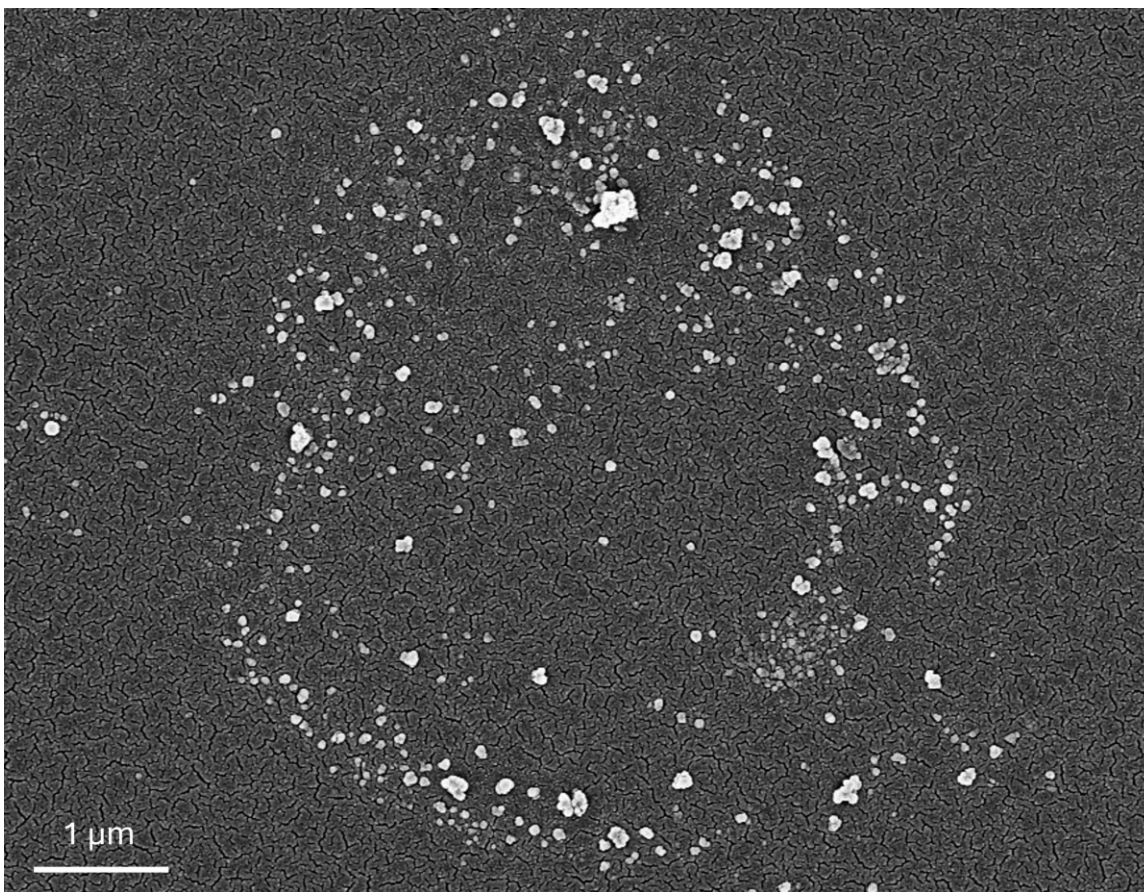
Assignment	Band position (cm^{-1})				
	mPEG	PLA	PCL	PEG- <i>b</i> -PLA	PEG- <i>b</i> -PCL
O-H	3450	-	-	-	-
C-H stretch	2875	2995	2943	2871, 2995	2893, 2943
C=O	-	1753	1720	1749	1722
CH ₂ bending	1466	-	1469	-	1469
CH ₃ bending	-	1454	-	1452	1419
C-O-C	1095	1182	1160	1182	1178
C-O stretch	1059	1086	1107	1080	1103



Appendix Figure 8. ^1H -NMR spectrum of ϵ -caprolactone in CDCl_3 .



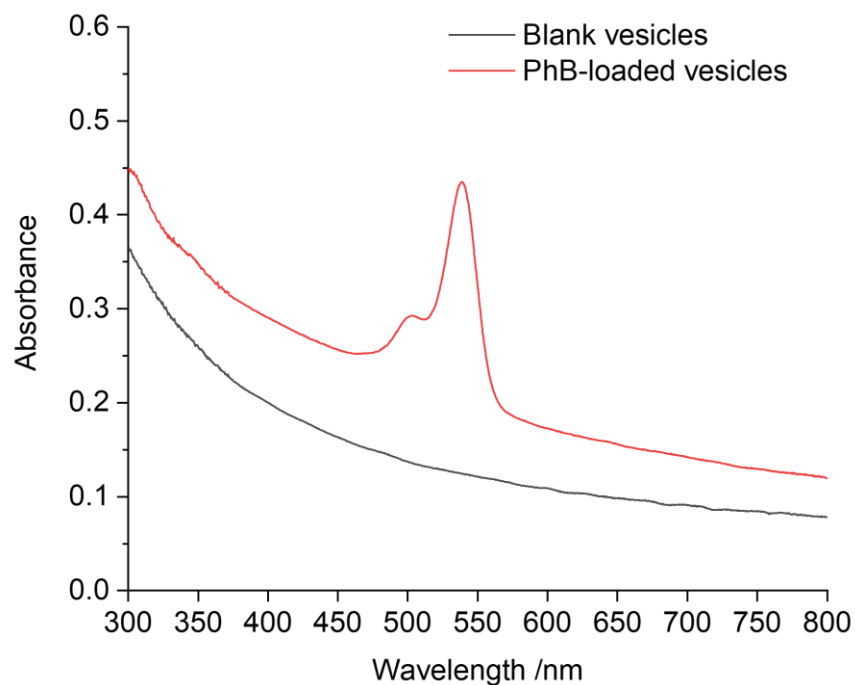
Appendix Figure 9. ^1H -NMR spectra of PEG₁₁₄-*b*-PCL _{x} in CDCl₃, with $x = 100, 150, 300$ and 500 .



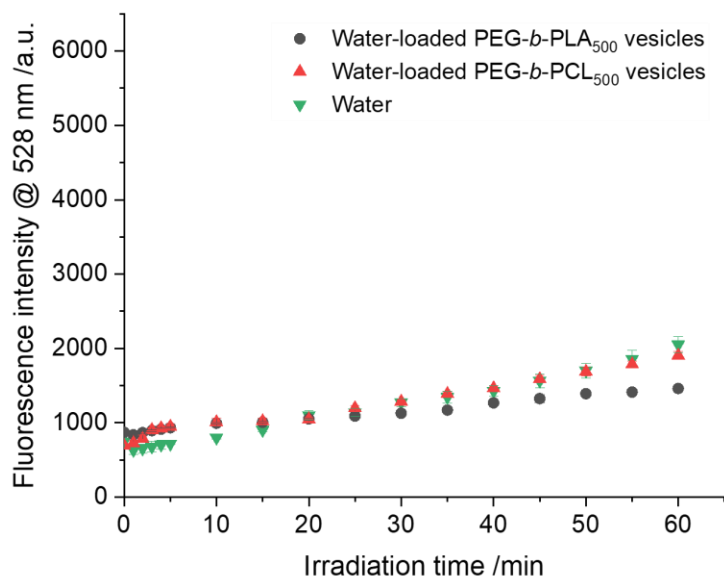
Appendix Figure 10. Coffee-ring effect upon drying of a drop-cast PEG₁₁₄-*b*-PCL₅₀₀ vesicle suspension onto a Si wafer, observed in the FESEM.



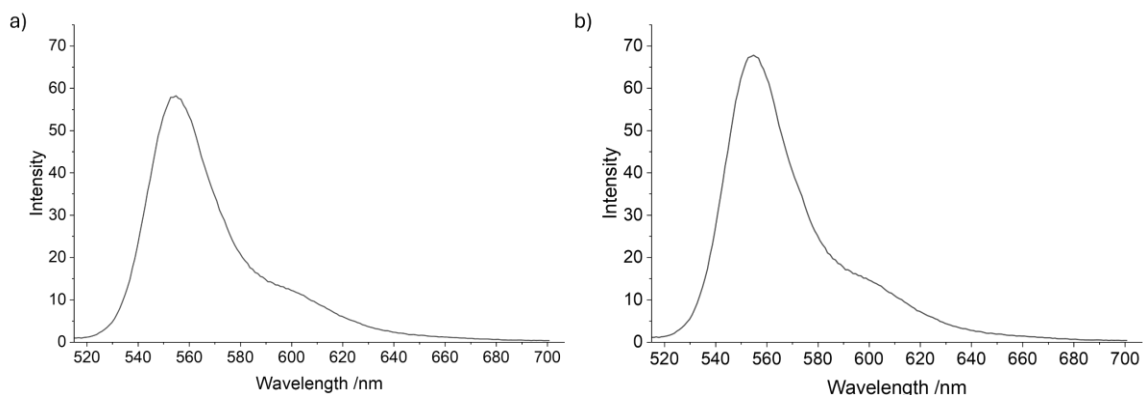
Appendix Figure 11. Epifluorescence microscopy image of water-loaded PEG-*b*-PLA₅₀₀ vesicles in a) bright field, b) emission resulting from a filter set with $\lambda_{\text{ex}} = 540\text{--}552\text{ nm}$ and $\lambda_{\text{em}} > 590\text{ nm}$ and c) bright field and emission images are merged to localise the PhB. Image shows pseudo-colouring of the fluorescence emission. Scale bars: 20 μm .



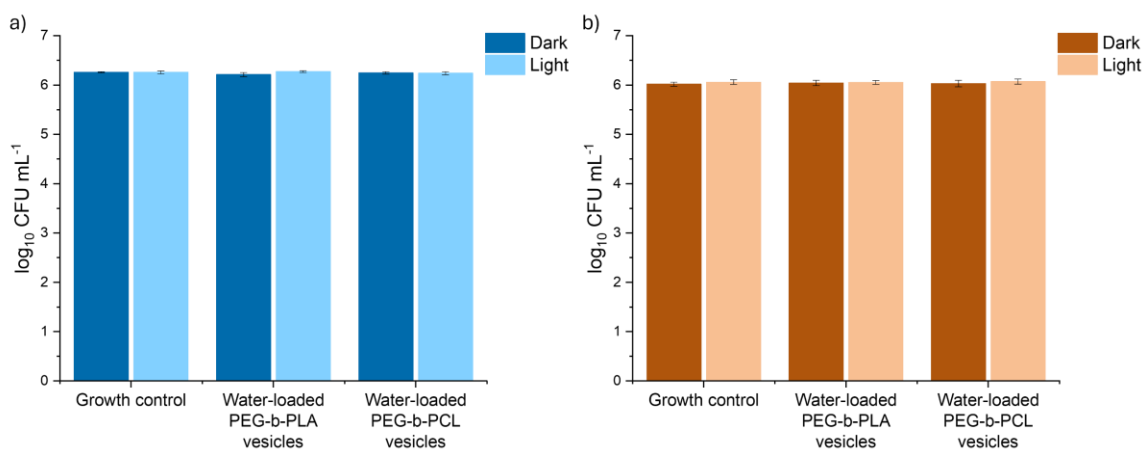
Appendix Figure 12. UV-Vis spectra of water- and PhB-loaded PEG-*b*-PLA₅₀₀ vesicles.



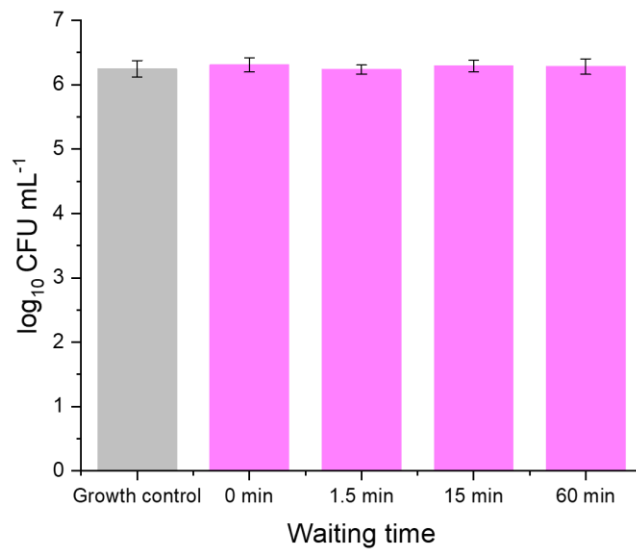
Appendix Figure 13. SOSG probe signal to detect ¹O₂ production of neat PhB, PhB-loaded vesicle suspensions and water with increasing irradiation times ($\lambda = 520$ nm, 4 mW cm⁻²). The errors bars represent the standard deviation (n = 3).



Appendix Figure 14. Fluorescence emission spectra of PhB-loaded a) PEG₁₁₄-b-PLA₅₀₀ and b) PEG₁₁₄-b-PCL₅₀₀ vesicle suspensions ($\lambda_{\text{ex}} = 504 \text{ nm}$). All suspensions were measured at 5% v/v concentration in Milli-Q water.

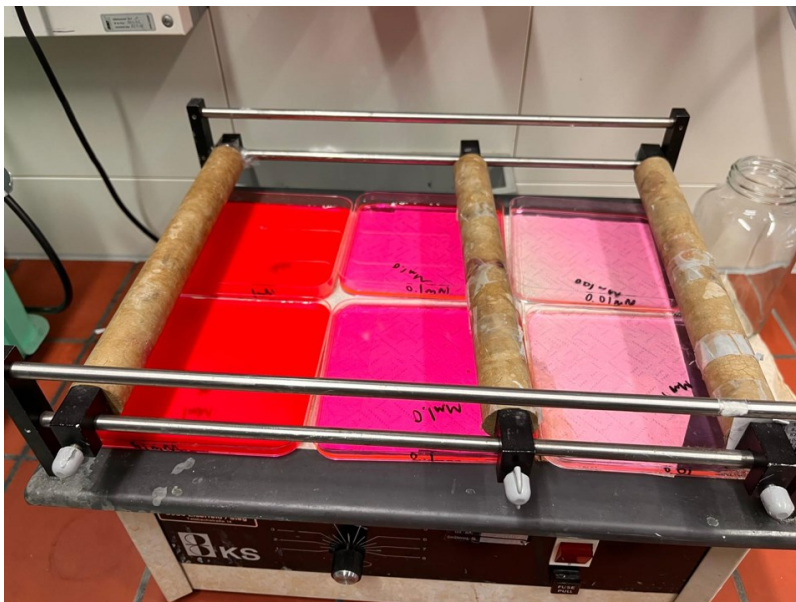


Appendix Figure 15. Antimicrobial activity of water-loaded vesicles against a) *S. aureus* RN4220 and b) *P. aeruginosa* ATCC 19660 at a concentration of 10^6 CFU mL^{-1} . The samples were irradiated for 15 min ($\lambda=520 \text{ nm}$, 4 mW cm^{-2}) (Light) or incubated in the dark for 15 min (Dark). The error bars correspond to the standard deviation of biological replicas (n = 3).

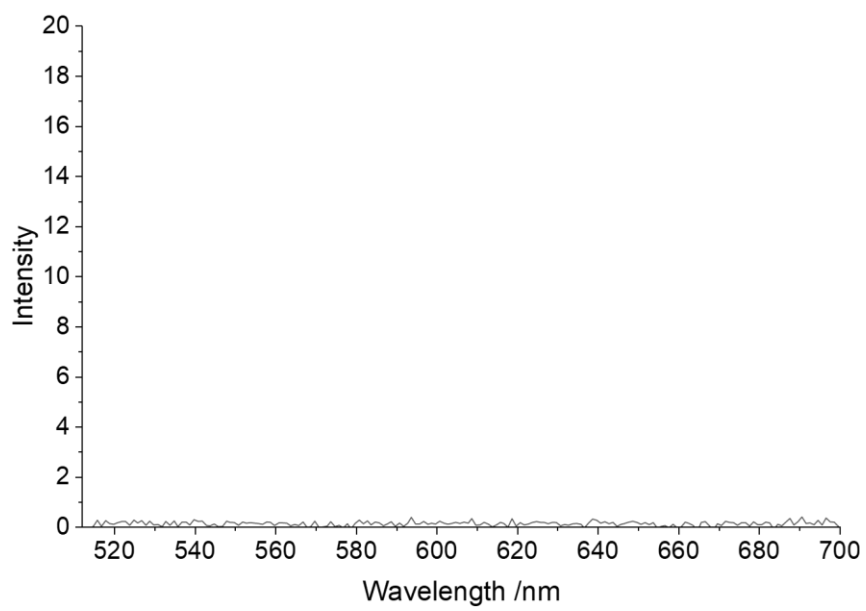


Appendix Figure 16. PhB-loaded PEG-*b*-PLA₅₀₀ with *S. aureus* RN4220 10^6 CFU mL^{-1} in LB. Samples were irradiated for 15 min, then a waiting time was observed before adding the bacteria.

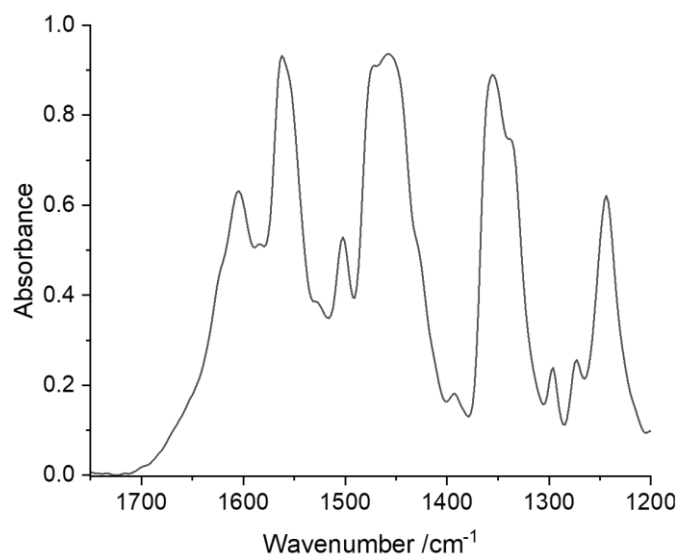
9.3. Chapter 6 appendix



Appendix Figure 17. Photograph of PhB-CS film preparation whilst modifying with the PhB NHS ester solution of varying PhB concentrations.



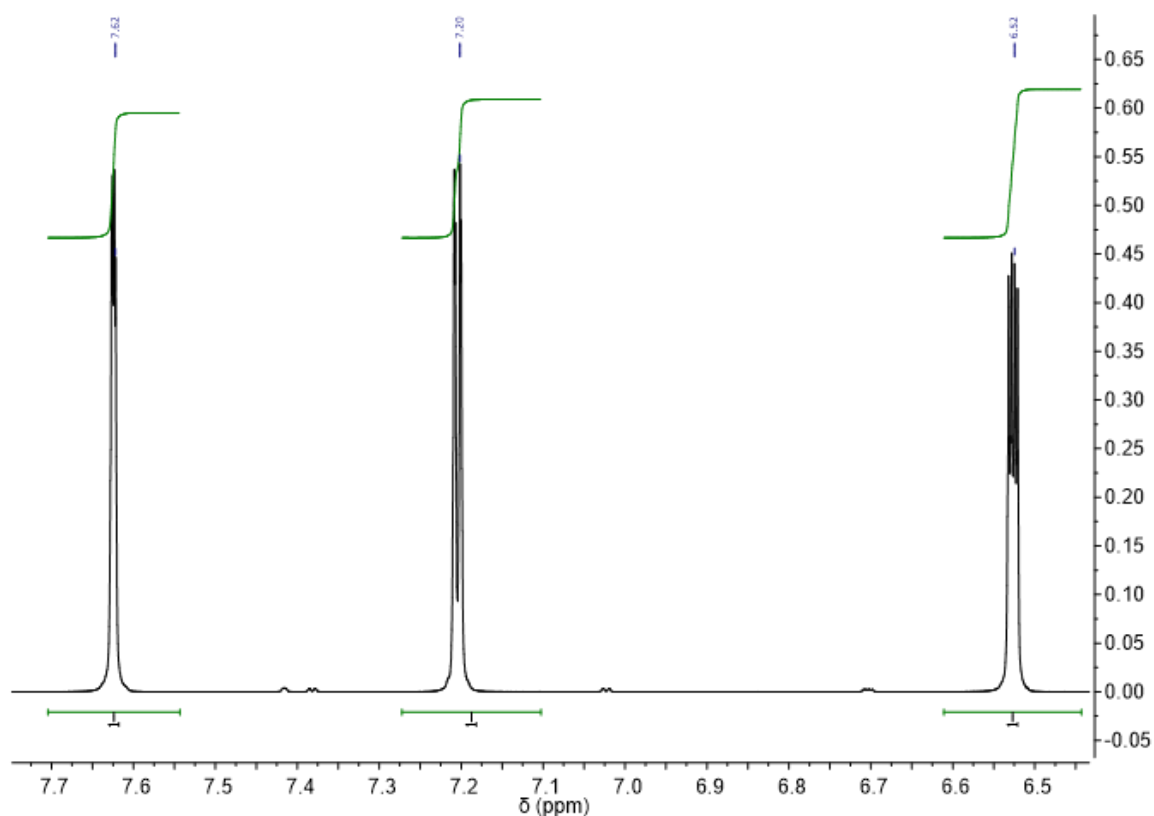
Appendix Figure 18. Fluorescence emission spectrum ($\lambda_{\text{ex}} = 504 \text{ nm}$) of eluent (20% EtOH) after extensive washing of 1.00 mM PhB-CS films.



Appendix Figure 19. ATR-FTIR spectrum of Phloxine B.

Appendix Table 2. ATR-FTIR band assignments for PhB.

Band position (cm ⁻¹)	Assignment
1230	C-O stretching (xanthene ether)
1260	C-O stretching (aryl-O)
1300	C-O stretching & aromatic C-C
1340	COO ⁻ symmetric stretching
1355	Aromatic C-Cl stretching
1390	COO ⁻ symmetric stretching
1450	Aromatic C-C stretching
1458	Aromatic ring vibration (C=C)
1500	Aromatic skeletal vibration
1540	Aromatic C=C stretching
1560	COO ⁻ asymmetric stretching
1590	Aromatic C=C stretching
1610	Aromatic C=C stretching
1620	C=O stretching



Appendix Figure 20. ¹H-NMR spectrum of PhB in D₂O.

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