

Rapid Detection and Photodynamic Eradication of Bacteria: A Dual Approach to Combat Multidrug-Resistant Bacterial Infections

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Abbreviations and acronyms

AMR	Antimicrobial resistance
ATR	Attenuated Total Reflectance
aPDT	Antimicrobial photodynamic therapy
BSA	Bovine serum albumin
β -Gus	β -Glucuronidase
CS	Chitosan
CF	Cystic Fibrosis
cm	Centimeters
CFU	Colony forming unit
CD ₃ COOD	Deuterated acetic acid
D ₂ O	Deuterated water
DMSO	Dimethyl sulfoxide
<i>E. coli</i>	<i>Escherichia coli</i>
EDC.HCl	<i>N</i> -(3-Dimethylaminopropyl)- <i>N'</i> -ethylcarbodiimide Hydrochloride
eV	Electron Volt
FTIR	Fourier Transform Infrared Spectroscopy
h	Hour
I _F	Fluorescence intensity
kV	Kilovolt
LB	Lysogeny broth
LOD	Limit of detection
LOQ	Limit of quantification
LRF	Log reduction factor
4-MU	4-Methylumbelliferone
MUG	4-Methylumbelliferyl- β -D-glucuronide
MDR	Multi-drug resistant
min	Minutes
mol	Moles
mL	Milliliter
mM	Millimoles per Liter
NHS	<i>N</i> -Hydroxy succinimide
NMR	Nuclear Magnetic Resonance
nm	Nanometer
nM	Nanomoles per liter

PBS	Phosphate buffered saline
PEO	Poly(ethylene) oxide
PS	Photosensitizer
PA	<i>Pseudomonas aeruginosa</i>
RGB	Red-Green-Blue
rpm	Rotations per minute
SEM	Scanning Electron Microscopy
SOSG	Singlet oxygen sensor green
SA	<i>Staphylococcus aureus</i>
wt%	Weight percent
XPS	X-ray Photoelectron Spectroscopy
X-Gluc	5-bromo-4-chloro-3-indolyl- β -D-Glucuronide
°C	Degree Celsius
μ L	Microliter
μ M	Micromoles per liter

Abstract

Antimicrobial resistance (AMR) represents a growing global health threat, driven in part by the overuse of broad-spectrum antibiotics and the limited effectiveness of conventional diagnostics and therapeutic approaches. This Thesis addresses a dual strategy to address these challenges: (i) rapid, enzyme-responsive diagnostics to enable early detection and mitigate antibiotic misuse, and (ii) targeted antimicrobial photodynamic therapy (aPDT) to enhance treatment efficacy. For bacterial detection, the strategy of autonomously reporting chitosan-based sensors was further developed, focusing on *E. coli* detection using β -glucuronidase as a biomarker. A paper-based carrier for chitosan hydrogels was integrated with smartphone-assisted RGB analysis, enabling visible, low-cost, and user-friendly point-of-care diagnostics. The system relies on color intensity changes of a released reporter dye, modulated by enzyme concentrations corresponding to bacterial load. To improve β -glucuronidase sensitivity and facilitate scalable fabrication, enzyme-responsive chitosan-based electrospun nanofibers were investigated. At a fixed enzyme concentration, these nanofibers exhibited a 3.4-fold increase in the initial enzymatic hydrolysis rate compared to the hydrogel system and achieved a limit of detection of 4.7 nM after 60 min, in contrast to 16 nM for the hydrogel system. The enhanced sensitivity of the nanofiber platform was further validated through *in vitro* experiments using pathogenic *E. coli*, demonstrating its potential utility in real biological samples. For bacterial eradication, a Ru(II)-based photosensitizer (Ru(phen)₃) encapsulated in polymeric nanocarriers was evaluated against *P. aeruginosa* in its planktonic form, in biofilms and in artificial sputum medium (ASM), which simulates the Cystic Fibrosis (CF) lung environment. Ru(phen)₃-loaded vesicles exhibited a greater photodynamic inactivation of planktonic *P. aeruginosa* compared to Ru(phen)₃-loaded micelles, attributed to the higher loading capacity of Ru(phen)₃. The biological complexity of ASM significantly influenced aPDT efficacy, with Ru(phen)₃-loaded vesicles showing reduced activity compared to the neat Ru(phen)₃ at similar overall Ru(phen)₃ concentrations. In biofilm eradication assay, a 4.8 log reduction was achieved with neat Ru(phen)₃ while Ru(phen)₃-loaded vesicles resulted in 2 log reduction after 120 min of irradiation. Notably, prolonged pre-incubation times appeared to facilitate deeper photosensitizer penetration into biofilms, thereby improving aPDT outcomes. This approach was further applied to multidrug-resistant (MDR) isolates of *P. aeruginosa* and *S. aureus* from CF patients, as well as pathogenic *E. coli*. The results underscore the therapeutic potential and versatility of Ru(phen)₃-loaded vesicles in treating MDR infections, particularly those associated with CF pathology. Overall, this Thesis contributes to the development of effective, enzyme-responsive diagnostic tools and optimized aPDT treatment strategies for combating bacterial infections to fight AMR.

Zusammenfassung

Antimikrobielle Resistenzen (AMR) stellen eine zunehmende globale Bedrohung für die allgemeine Gesundheit dar, die unter anderem durch den übermäßigen Einsatz von Breitbandantibiotika sowie die begrenzte Wirksamkeit konventioneller diagnostischer und therapeutischer Ansätze begünstigt wird. Diese Dissertation verfolgt eine zweigleisige Strategie zur Bewältigung dieser Herausforderungen: (i) schnelle, enzymbasierte Diagnostik zur frühzeitigen Erkennung und zur Verringerung des unsachgemäßen Antibiotikaeinsatzes sowie (ii) gezielte antimikrobielle photodynamische Therapie (aPDT) zur effizienten Behandlung.

Zur Detektion von Bakterien wurde die Strategie chitosanbasierter autonomer Sensormaterialien weiterentwickelt, mit besonderem Fokus auf der Detektion von *E. coli* unter Verwendung von β -Glucuronidase (β -GUS) als Biomarker. Ein papierbasierter Träger für Chitosan-Hydrogele wurde mit einer Smartphone-gestützten RGB-Analyse kombiniert, was eine visuelle, kostengünstige und benutzerfreundliche Diagnostik am Point-of-Care ermöglichte. Das System basiert auf Farbintensitätsänderungen eines freigesetzten Farbstoffs, die durch enzymatische Aktivität in Abhängigkeit von der bakteriellen Belastung moduliert werden. Zur Verbesserung der Empfindlichkeit gegenüber β -GUS und zur Skalierbarkeit der Herstellung wurden enzymreaktive, elektrogesponnene Chitosan-Nanofasern untersucht. Bei konstanter Enzymkonzentration zeigten diese Nanofasern eine 3,4-fach höhere anfängliche Hydrolyserate im Vergleich zum Hydrogelsystem und erreichten eine Nachweisgrenze von 4,7 nM nach 60 Minuten, im Vergleich zu 16 nM beim Hydrogelsystem. Die verbesserte Empfindlichkeit der Nanofaserplattform wurde zudem durch In-vitro-Versuche mit pathogenen *E. coli*-Stämmen bestätigt, was ihre potenzielle Anwendbarkeit in realen biologischen Proben unterstreicht.

Zur Eradikation von Bakterien wurde ein Ru(II)-basierter Photosensibilisator (Ru(phen)_3), verkapselt in polymeren Nanoträgern, gegen *Pseudomonas aeruginosa* (*P. aeruginosa*) in planktonischer Form, in Biofilmen sowie in künstlichem Sputummedium (ASM) untersucht, das die Lungenumgebung bei Mukoviszidose (CF) simuliert. Ru(phen)_3 -beladene Vesikel zeigten eine stärkere photodynamische Inaktivierung planktonischer *P. aeruginosa* im Vergleich zu Ru(phen)_3 -beladenen Mizellen, was auf die höhere Beladungskapazität der Vesikel zurückgeführt wurde. Die biologische Komplexität des ASM beeinflusste die Wirksamkeit der aPDT erheblich; Ru(phen)_3 -beladene Vesikel zeigten bei gleicher Gesamtkonzentration eine geringere Aktivität als freies Ru(phen)_3 . Im Biofilm-Eradikationsversuch wurde mit freiem Ru(phen)_3 eine Reduktion von 4,8 Log-Stufen erzielt,

während Ru(phen)₃-beladene Vesikel nach 120 Minuten Bestrahlung eine Reduktion von 2 Log-Stufen erreichten. Auffällig war, dass längere Vorinkubationszeiten offenbar das tiefere Eindringen des Photosensibilisators in die Biofilme begünstigten und somit die Effektivität der aPDT verbesserten.

Dieser Ansatz wurde auch auf multiresistente (MDR) Isolate von *P. aeruginosa* und *S. aureus* aus CF-Patienten sowie auf pathogene *E. coli*-Stämme übertragen. Die Ergebnisse unterstreichen das therapeutische Potenzial und die Vielseitigkeit von Ru(phen)₃-beladenen Vesikeln bei der Behandlung multiresistenter Infektionen, insbesondere im Zusammenhang mit der CF-Pathologie. Insgesamt leistet diese Dissertation einen Beitrag zur Entwicklung effektiver, enzymreaktiver Diagnostiksysteme sowie zur Optimierung aPDT-basierter Therapieansätze zur Bekämpfung bakterieller Infektionen im Kampf gegen AMR.

1 Introduction and motivation

Antimicrobial resistance (AMR) is one of the most pressing global public health threats. In 2021, 4.71 million deaths were associated with bacterial AMR.¹ The WHO projects that this number could rise to 10 million annually by 2050.² AMR escalates healthcare costs due to the use of more expensive drugs, prolonged illness, and complex treatments. According to the World Bank, AMR could drive 28 million people into extreme poverty yearly by 2050 and cost the global economy up to USD 1 trillion per year.³

AMR is defined by the ability of bacteria, viruses, and other microorganisms to acquire or develop resistance against antimicrobial agents.⁴ This phenomenon significantly compromises the efficacy of standard treatments, increasing the risk of widespread infections, prolonged illness, and higher mortality. Recognising its severity, the World Health Organization (WHO) has declared AMR a global threat, urging the development of novel antibiotics and innovative therapeutic and diagnostic strategies.⁵

The prolonged and repetitive use of antibiotics has led to the emergence of multidrug-resistant (MDR) bacteria, which significantly limit the available therapeutic options.⁶ Among the most concerning MDR pathogens belong to the ESKAPE group: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter spp.*, which exhibit alarmingly high resistance profiles.⁷ Effective treatment of MDR infections requires extensive susceptibility screening for the available drug classes to circumvent resistance mechanisms.⁸ Among these pathogens, *Pseudomonas aeruginosa* and *Staphylococcus aureus* are of particular concern in patients with Cystic Fibrosis (CF), where they often co-exist and colonise the respiratory tract and are associated with chronic infections.⁹ Their ability to form robust biofilms^{10,11} enhances bacterial resistance against antibiotics and the host immune responses,¹² further complicating eradication efforts and exacerbating the AMR.

Another significant contributor to AMR is the prescription of broad-spectrum antibiotics without definitive diagnostics. Without rapid and accurate identification of the bacterial pathogen, clinicians often resort to broad-spectrum antimicrobials, which promotes the emergence and spread of resistant strains.^{13,14} Addressing this issue requires the development of rapid, sensitive, and specific bacterial detection platforms for timely and appropriate treatment, thus limiting the misuse and overuse of antibiotics. Consequently, there is a growing interest in the design of novel diagnostic platforms that can offer *in situ*, selective, and rapid detection of bacterial infections using simple and accessible clinical techniques.^{15,16}

Conventional bacterial detection techniques are typically culture-based methods, including agar plate culturing, Gram staining, microscopy, and molecular diagnostics such as polymerase chain reaction (PCR).^{17,18,19} Despite being the gold standard, these methods have several limitations: they are time-consuming, labour-intensive, and require specialised equipment and technical expertise. While PCR-based techniques offer high sensitivity, they are costly and often inaccessible in low-resource clinical settings.^{20,21,22,23} More advanced diagnostic tools, such as mass spectrometry, optical endomicroscopy, and confocal laser scanning microscopy using white light technology, offer improved accuracy in bacterial detection^{24,25,26} but their complexity, cost, and the requirement for trained personnel restrict their applicability in routine clinical use. Therefore, there is an urgent need for cost-effective, rapid, and user-friendly point-of-care (POC) diagnostic tools suitable for diverse healthcare settings, including those with remote or resource-limited areas.

To address these challenges, significant research has focussed on alternative detection strategies, particularly those utilizing nanotechnology. Nanomaterial-based platforms, including nanoparticles,^{27,28} nanocapsules,^{29,30} liposomes^{29,31}, and nanofibers,^{32,33,34} offer promising approaches for enhancing bacterial detection sensitivity and specificity. These platforms have been integrated into wound dressings and biosensors for real-time infection monitoring. One promising approach involves enzyme-responsive colorimetric sensors that detect bacteria based on the presence of enzymes secreted by specific pathogens.^{35,36,37,38,39} These sensors exhibit visible colour changes in response to enzymatic activity, allowing rapid and autonomous detection. Chitosan-based hydrogels have been widely investigated as colorimetric sensing materials for the rapid and selective detection and visual identification of bacteria.^{36,39} Paper-based microfluidic devices have also been developed to serve as carriers for reporting units for applications in health diagnostics, food safety, and environmental monitoring.⁴⁰ Recently, electrospun nanofibers have emerged as a promising tool for bacteria detection due to their high surface area-to-volume ratio, tunable porosity, and versatile functionalization capabilities.⁴¹ Among them, chitosan-based electrospun nanofibers have been reported for their antibacterial and biomedical applications.^{42,43,44} Several colorimetric sensors made from electrospun nanofibers, including poly(ethylene oxide) and polyurethane,⁴⁵ poly(vinyl alcohol-co-ethylene),⁴⁶ and polyurethane^{47,48} have been studied for detecting bacterial species, mainly non-pathogenic *E. coli*.

In parallel with the advances in diagnostics, alternative treatment strategies for MDR infections are also being actively explored. These include antimicrobial peptides,⁴⁹ bacteriophage therapy,⁵⁰ and quorum-sensing inhibitors.⁵¹ Among them, antimicrobial photodynamic therapy (aPDT) has gained attention due to the fast and efficient bactericidal effect and lower risk of resistance build-up.⁵² aPDT involves the activation of photosensitizers

(PS) by light in the presence of molecular oxygen, generating reactive oxygen species (ROS) that damage bacterial cells. One key advantage of aPDT is the localized, on-demand treatment of MDR infections. It was shown that aPDT possesses a very low risk of contributing to resistance build-up, since the bactericidal effect is caused by the action of ROS, which simultaneously targets multiple cellular structures and hence impede the resistance build-up.⁵² For many common MDR bacteria, aPDT has already been proven to be a strategy to avoid the build-up of resistances.^{53,54} Furthermore, among synthetic PS, metal complexes such as ruthenium (II) complexes (Ru(II)) have been extensively studied for PDT effects against fatal cancers, and some lead complexes undergo phase II clinical studies for PDT treatment of bladder cancer.^{55,56,57}

However, the clinical translation of aPDT is often limited by the physicochemical properties of many PS, such as limited biocompatibility, limited solubility in aqueous or biological media, dark toxicity, and reduced photoactivity in the aggregated states.^{58,59} To overcome these challenges, researchers have adopted drug delivery systems traditionally used for hydrophobic drugs. These include polymeric micelles, polymersomes, liposomes, and various nanoparticles, improving the solubility, stability, and biodistribution of PS.⁶⁰ A crucial design consideration for these carriers is their ability to remain in circulation without passively releasing the payload, followed by targeted and stimulus-responsive drug release at the site of infection. One commonly employed strategy is PEGylation, which enhances stabilized circulation within the body, as widely demonstrated in cancer therapeutics.⁶¹

This Thesis explores the optimization of established enzyme-responsive chitosan-based colorimetric sensors for developing diagnostic platforms for improved bacterial detection and investigates the polymeric nanocarriers loaded with Ru-based photosensitizers for the eradication of pathogenic bacteria using aPDT (Figure 1). Specifically, pathogenic and non-pathogenic *Escherichia coli* were detected using chitosan-based enzyme-responsive sensors targeting bacterial enzyme β -glucuronidase (β -Gus), while Ru-based PS encapsulated in polymeric nanocarriers were used to eradicate the planktonic form and biofilms of *Pseudomonas aeruginosa* isolated from CF patients. This approach was further expanded to eradicate bacteria in artificial sputum medium (ASM) mimicking the CF lung environment, and finally against other MDR bacteria.

Chapter 3 focuses on the development of an enzyme-responsive point-of-care diagnostic system by integrating a paper-based chitosan hydrogel with smartphone-based RGB analysis for the detection of *E. coli*. The enzyme cleavage of sensing substrate results in dye release, with signal intensity correlating to enzyme concentration and hence bacterial

load. The captured images under varying conditions form a comprehensive dataset for rapid, field-deployable bacterial diagnostics.

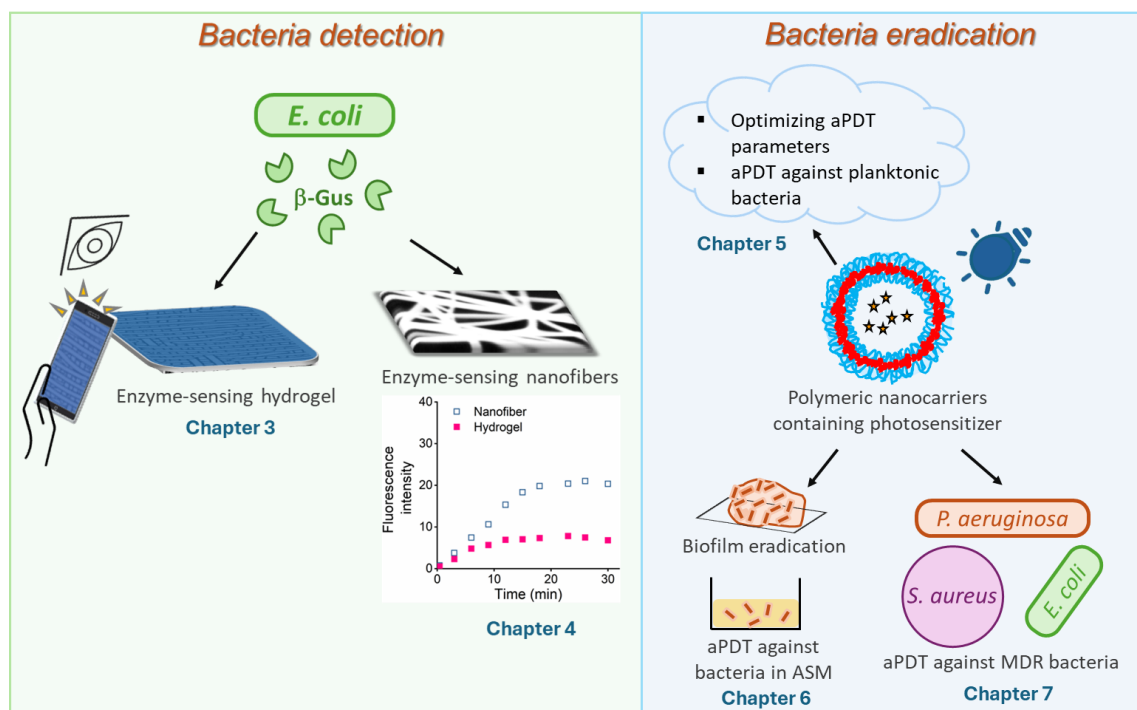


Figure 1: Schematic representation of the workflow in this Thesis: Bacterial detection using enzyme-responsive sensing hydrogels and nanofibers, and eradication of clinically relevant strains using aPDT with PS loaded nanocarriers.

To enhance sensitivity, Chapter 4 describes the fabrication of enzyme-responsive chitosan-based nanofibers via electrospinning. These nanofibers offer a high surface area and improved interaction with bacterial enzymes. Functionalised with fluorogenic β -Gus substrates, these nanofibers enabled the sensitive detection of a clinically relevant *E. coli* strain listed among WHO priority pathogens.

Chapter 5 evaluates the aPDT efficacy of Ru-based photosensitizers against planktonic *P. aeruginosa*. Commercially available Ru(phen)₃ complex was encapsulated in various polymeric carriers, and optimal conditions for light-mediated inactivation of *P. aeruginosa* CF isolate were reported. In Chapter 6, the study extends to examine the efficacy of Ru(phen)₃-loaded nanocarriers and free PS in ASM and biofilms. Finally, Chapter 7 broadens the investigation to include other MDR pathogens, such as a series of *P. aeruginosa*, *S. aureus* collected from CF patients, and pathogenic *E. coli*. The results highlight the therapeutic efficacy and versatility of Ru(phen)₃-loaded nanocarriers in treating MDR infections, particularly those relevant to CF pathology.

This Thesis presents a comprehensive approach to address two critical challenges in AMR management: rapid detection and effective treatment of MDR bacterial infections. By combining smart diagnostic materials and advanced therapeutic strategies, this work may in the long run contribute to the global fight against AMR.

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2 State of the art

2.1 Bacteria and bacterial biofilms

Bacteria are single-celled prokaryotic microorganisms that lack a nuclear membrane, are metabolically active, and divide by binary fission.¹ They colonize virtually every habitat on Earth, from soil and aquatic environments to extremes of temperature and pressure.^{2,3} While many species are beneficial for ecological functions such as nitrogen fixation⁴, digestion⁵, and biotechnological functions⁶, others are pathogenic, causing diseases in humans,⁷ animals,⁸ and plants.⁹ Their rapid reproduction and physiological adaptability make them contributors to ecosystems and persistent challenges in clinical settings.

In both environmental and clinical contexts, bacteria predominantly do not exist as isolated, planktonic forms, but tend to congregate on surfaces and embed themselves within a self-produced extracellular polymeric substance (EPS), forming highly structured communities known as biofilms.¹⁰ The transition from the planktonic state to biofilm growth occurs as a consequence of environmental changes that trigger the dysregulation of multiple regulatory networks. Thus, upon sensing a stress signal, free-living (planktonic) cells will initiate attachment to a surface which will lead to the formation of a biofilm that has a greater ability to withstand environmental challenges.¹¹ Biofilm development occurs in distinct stages, as shown in Figure 1.

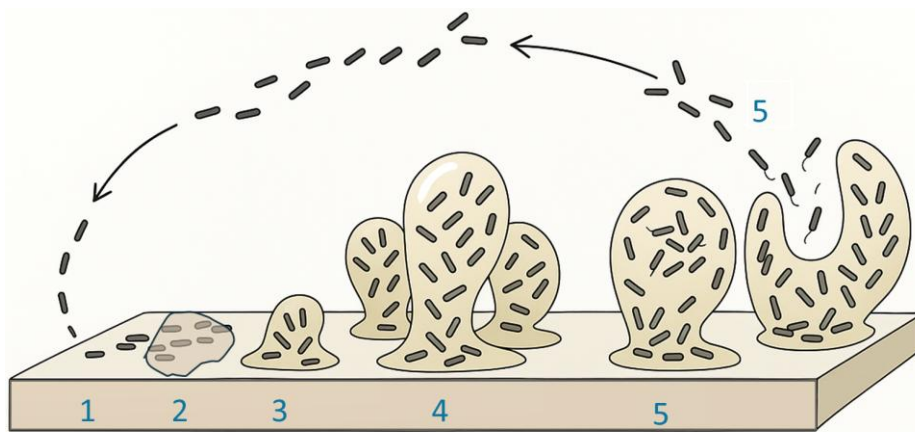


Figure 1: Diagram showing the five-stage process of biofilm development. Stage 1: initial reversible attachment of planktonic cells to a surface. Stage 2: production of EPS resulting in more firmly adhered irreversible attachment. Stage 3: early development of biofilm architecture. Stage 4: maturation of biofilm architecture. Stage 5: dispersion of single cells from the biofilm (adapted with permission from Stoodley, P.; Sauer, K.; Davies, D.G.; Costerton, J.W. Biofilms as complex differentiated communities. *Annu. Rev. Microbiol.* **2002**, 56, 187-209).¹²

The EPS matrix is mainly composed of polysaccharides, proteins, nucleic acids, and lipids. It facilitates adhesion to surfaces, nutrients and water retention, and mechanical stability while protecting against environmental stresses.¹³

2.1.1 Bacteria classification based on the Gram staining method

Based on the Gram staining technique, bacteria can be classified into two main groups: Gram-positive and Gram-negative. This classification is based on differences in how bacterial cell walls interact with a primary stain, usually crystal violet. When observed under a microscope, bacteria that retain the violet stain appear purple-brown are identified as Gram-positive. By contrast, those that do not retain the stain and instead take up a counterstain, appearing red, are classified as Gram-negative.¹⁴ Figure 2 shows the structural differences between the cell walls of Gram-positive and Gram-negative bacteria.

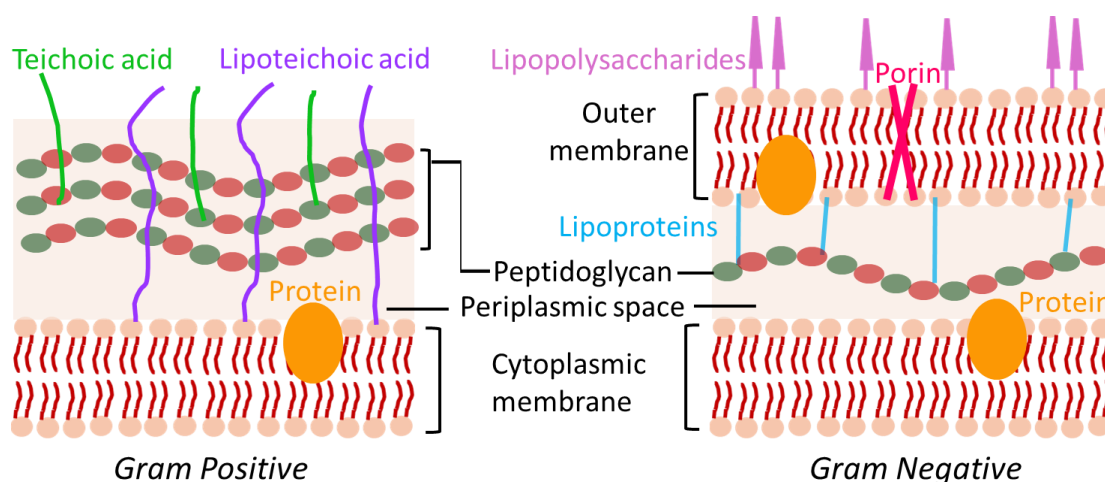


Figure 2: Structural differences in cell walls of Gram-positive and Gram-negative bacteria (adapted with permission from Technology Networks. Available online: <https://www.technologynetworks.com/immunology/articles/gram-positive-vs-gram-negative-323007> (accessed on 28 March 2025)).¹⁵

Gram-positive bacteria are characterized by the absence of an outer membrane and the presence of a thick peptidoglycan layer. This layer contains long-chain anionic polymers such as teichoic acids, which are bonded to the peptidoglycan, and lipoteichoic acids, which are linked to membrane lipids. On the other hand, Gram-negative bacteria feature a relatively thin peptidoglycan layer, which is encased by an outer membrane enriched with lipopolysaccharides.¹⁶ In Gram staining method, initially all bacteria take up crystal violet dye. Upon solvent treatment, the outer lipid layer membrane of Gram-negative bacteria is dissolved, allowing the dye to be washed out, while, in Gram-positive bacteria the solvent causes dehydration of the thick peptidoglycan layer, effectively preventing the violet-iodine complex from diffusing out, which results in bacteria retaining the stain.¹⁴ As a preliminary diagnostic technique, Gram staining plays a vital role in the clinical setting. It assists healthcare professionals in the early identification of bacterial types, guides for suitable antibiotic

treatments and further diagnostic testing of infections such as pneumonia, urinary tract infections, and meningitis.^{14,17}

Escherichia coli

Escherichia coli (*E. coli*) is a Gram-negative, rod-shaped bacterium that naturally inhabits the intestines of humans and other warm-blooded animals.¹⁸ While the majority of *E. coli* strains are harmless and function as part of the normal gut microbiota, certain pathogenic strains such as Shiga toxin-producing *E. coli* can lead to serious foodborne illnesses.¹⁹ When these pathogenic strains are ingested; mainly from contaminated food, raw or undercooked ground meat, unpasteurized milk, and raw produce like vegetables and sprouts; they can result in gastrointestinal illnesses in humans.^{19,20} Beyond the gut, it is a common opportunistic pathogen causing extraintestinal infections when introduced to other parts of the body. These include urinary tract infections (UTIs), pneumonia, and bloodstream infections.^{21,22} The pathogenic potential of *E. coli* is largely due to its ability to bypass the host immune responses and develop resistance to multiple antibiotics.²⁰

Pseudomonas aeruginosa

Pseudomonas aeruginosa (*P. aeruginosa*) is a Gram-negative, aerobic, motile bacterium with a slightly curved rod shape and is widely distributed in various environments, including air, water, soil, and the tissues of plants and animals.²³ Recognized as a multidrug-resistant opportunistic pathogen, *P. aeruginosa* is capable of causing a wide range of serious acute and chronic infections. These include pneumonia, urinary tract infections, otitis media, meningitis, and are particularly severe in individuals with cystic fibrosis.^{24,25} The ability of *P. aeruginosa* to cause disease is attributed to its extensive arsenal of virulence factors and extraordinary genetic adaptability, which enables it to survive in diverse environments and evade the immune system defence.²⁶ One of the most challenging aspects of *P. aeruginosa* infections is its ability to form biofilms. In this state, the bacterium can endure hypoxic conditions or harsh environments.²⁷ Biofilm formation also enhances its resistance to antibiotics and contributes to persistent infections. The rapid mutation rate and adaptability of *P. aeruginosa* further complicate treatment, often leading to antibiotic resistance.²⁸ Moreover, *P. aeruginosa* is a leading cause of hospital-acquired infections and is frequently associated with contaminated medical equipment, such as ventilators. Its preference for moist surfaces allows it to thrive on medical devices, increasing the risk of infections in healthcare settings.²⁹

Staphylococcus aureus

Staphylococcus aureus (*S. aureus*) is a Gram-positive, spherical bacterium that can grow under both aerobic and anaerobic conditions. It exists as both a harmless commensal

organism and a formidable opportunistic pathogen, capable of causing a wide array of human diseases.³⁰ Clinically, it is one of the most important bacterial pathogens, responsible for causing soft tissue infections and life-threatening illnesses such as pneumonia, endocarditis, osteomyelitis, septic arthritis, and bacteremia.³¹ *S. aureus* colonizes the skin, mucous membranes, and nasal passages. It can transition from a benign presence to a dangerous invader, especially when host barriers are compromised or immune responses are weakened. Its pathogenic versatility is largely due to an extensive arsenal of virulence factors that facilitate adherence to host tissues, immune evasion, and cellular damage.³⁰ The ability of *S. aureus* to form resilient biofilms on implanted medical devices and prosthetic materials significantly contributes to its persistence in the host. These biofilms provide protection against both antimicrobial treatments and immune system attacks, often leading chronic or recurrent infections.³² Moreover, the emergence and global spread of methicillin-resistant *S. aureus*, both in hospitals and in the community, have severely limited therapeutic options. This resistance contributes to increased rates of disease severity, treatment failure, and mortality worldwide.³¹

2.1.2 Bacterial enzymes

Enzymes, also known as biocatalysts, accelerate biochemical reactions by lowering the activation energy required for the transformation of substrates into products. While naturally occurring in living organisms, enzymes can also be isolated and employed in various industrial and biotechnological processes due to their catalytic efficiency and specificity.³³ The most commonly targeted biomarkers in bacterial detection are hydrolytic enzymes. These include alkaline phosphatase, β -galactosidase, β -glucuronidase, and β -glucosidase, which act specifically on phosphoric acid, galactoside, glucuronide, and glucoside substrates respectively.³⁴ There is a growing interest in developing simple, on-site detection methods for pathogenic bacteria, such as *E. coli*, particularly approaches suitable for use by non-specialists outside of laboratory settings. *E. coli* expresses several enzymes that can serve as effective biomarkers. Among these, β -glucuronidase is particularly notable due to its broad presence in approximately 95% of *E. coli* strains so it is best known as a characteristic enzyme for *E. coli* detection.³⁵

β -glucuronidase for *E. coli* detection

The β -glucuronidase is a tetrameric protein with an approximate molecular weight of 290 kDa, an isoelectric point (pI) of 4.8, and optimal catalytic activity observed at a pH range of 6.0 to 7.0.³⁶ β -glucuronidase (EC 3.2.1.31) is a glycoside hydrolase that catalyzes the hydrolysis of β -linked D-glucopyranosiduronic acids (β -glucuronides) to glucopyranosiduronic acid (glucuronic acid) and the corresponding aglycone (Figure 3).³⁷

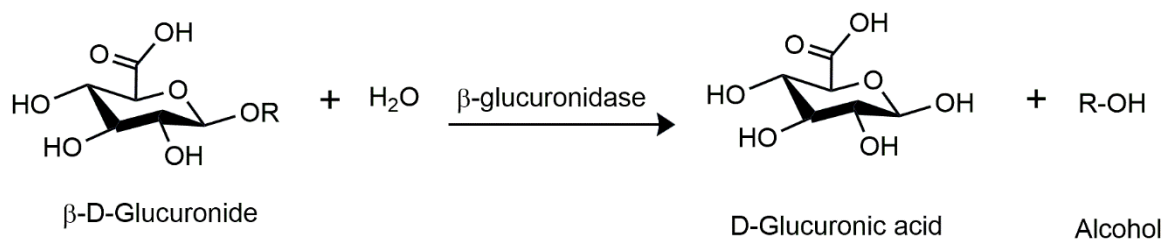
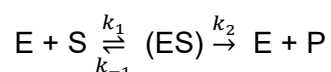


Figure 3: Hydrolysis of β -D-glucuronide compound into D-glucuronic acid and an alcohol, catalyzed by the enzyme β -glucuronidase.

β -glucuronidase activity can be assessed using various chromogenic or fluorogenic substrates through enzyme-catalysed reactions.

2.1.3 Enzyme-catalysed reactions

In an enzyme-catalysed reaction, substrate molecules (S) bind to the active site or catalytic site of the enzyme (E) and form an intermediate enzyme-substrate complex (ES). The active site of the enzyme interacts with specific substrates through non-covalent interactions such as hydrogen bonds, ionic bonds, or hydrophobic interactions.³⁸ Once the enzyme-substrate complex is formed, the enzyme facilitates the conversion of the substrate into the product (P). The product is then released from the enzyme due to a reduced binding affinity, allowing the enzyme to participate in subsequent catalytic cycles. The kinetics of enzyme-catalysed reactions can be quantitatively described using the Michaelis-Menten model.³⁹ The reaction scheme can be written as:



where k_1 and k_{-1} are the forward and reverse rate constants for substrate binding, and k_2 is the catalytic rate constant for product formation. Figure 4 shows the relation between the rate of catalysis and substrate concentration in the enzyme-catalysed reactions.³⁹

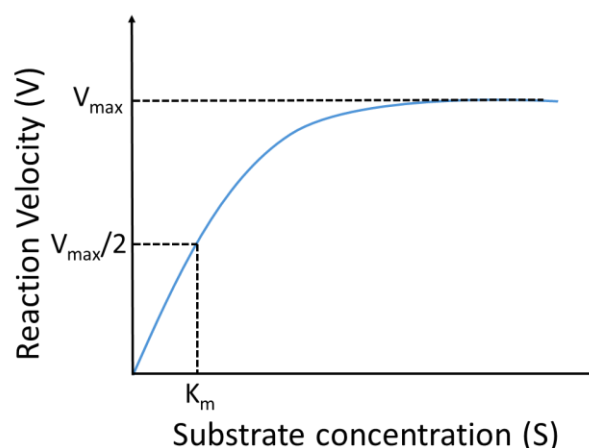


Figure 4: The rate or velocity of an enzyme-catalysed reaction as a function of substrate concentration according to the Michaelis-Menten equation.

At low substrate concentrations, the reaction rate/velocity increases linearly with [S]. However, as the substrate concentration continues to increase, the effect on reaction rate starts to diminish until a stage is reached ('plateau') where further increasing [S] has little effect on the reaction catalysis. This stage indicates the enzyme is approaching saturation with substrate and shows its maximum velocity, V_{max} . The Michaelis-Menten equation describes the relationship between the rate of catalysis V , and the substrate concentration [S] as:

$$V = \frac{V_{max} [S]}{K_M + [S]}$$

where $V_{max} = k_2 [E]_T$ and $[E]_T$ is the total enzyme concentration, V is defined as the number of moles of product formed per unit time, V_{max} is the maximum velocity of reaction, and K_M is the Michaelis-Menten constant, which is the substrate concentration at half of maximum velocity ($V_{max}/2$). K_M is defined as the substrate concentration at which half of the enzyme's active sites are occupied. It is a key parameter that reflects the affinity of an enzyme for its substrate; a lower K_M value indicates higher substrate affinity, as a smaller substrate concentration is required to achieve half-maximal enzyme activity. K_M depends on temperature, pH, and ionic strength. For $[S] \ll K_M$, $V = (V_{max}/K_M) [S]$; i.e. the initial rate V becomes directly proportional to [S]. On other hand, for $[S] \gg K_M$, $V = V_{max}$; i.e. the rate is maximum and independent of the concentration of substrate.^{39,33}

2.2 Biosensors for rapid bacteria detection

2.2.1 Biosensors

Biosensors are analytical devices that integrate a biological recognition element, such as enzymes, antibodies, nucleic acids, or cells, with a physicochemical transducer to detect target analytes.⁴⁰ The biological element interacts with the analyte using the lock-and-key principle of enzyme complex, and the transducer converts this interaction into a measurable signal. The concept of biosensors was first introduced by Clark and Lyons in 1962 with the development of a glucose enzyme electrode.⁴¹

2.2.2 Enzymatic colorimetric biosensors

Colorimetric biosensors are analytical devices that detect and quantify biological or chemical analytes through visible color changes. These sensors are based on specific interactions between the target analyte and a recognition element, which triggers a chemical or enzymatic reaction leading to a measurable colorimetric response. The resulting color change can be assessed either visually or using spectrophotometric techniques.⁴² The intensity of the color change is proportional to the concentration of the analyte, allowing for quantitative analysis.

Calibration curves derived from known analyte concentrations facilitate the accurate determination of unknown sample concentrations.⁴³

The integration of colorimetric assays with enzyme-catalyzed reactions has gained significant attention due to its operational simplicity and ease of visual interpretation.⁴⁴ Enzymatic colorimetric detection is based on a color change resulting from a specific chemical reaction between a substrate and its corresponding enzyme. Enzyme substrates used in colorimetric biosensing, developed in the 1970s,⁴⁵ have greatly simplified bacterial detection by producing visible signals when hydrolyzed by bacterial enzymes. Due to their strong ability to differentiate and visualize bacterial colonies, chromogenic substrates have significantly simplified microbiological evaluations, particularly for assessing bacterial metabolic activity and detecting pathogenic strains in food.^{34,46}

Enzyme substrates for bacterial enzyme detection

The identification of bacterial species can be effectively achieved using synthetic enzyme substrates designed to target species-specific enzymatic activities.⁴⁷ These substrates are typically incorporated into either liquid or solid growth media, where they interact with marker enzymes produced by the bacteria. Chromogenic media use synthetic enzyme substrates that change color when broken down by specific bacterial enzymes. This helps selective identification of pathogens based on their enzyme activity. Since enzyme activity is rarely exclusive to a single species, these media often incorporate multiple enzyme substrates or selective agents to improve specificity.⁴⁸ Most chromogenic formulations are both selective and differential, suppressing the growth of non-target microbes through agents like antibiotics, while enabling target organisms to metabolize chromogenic substrates and form distinctly colored colonies. This distinct coloration enhances the visibility of pathogens against background flora, which can increase detection accuracy and speed up diagnostic reporting. This visual distinction simplifies identification by reducing the number of colonies that require further testing in mixed cultures.⁴⁸ For instance, the CHROMagar™ provides a broad range of commercially available chromogenic media for selective and visual identification of various bacterial species⁴⁹ which have been developed to improve the sensitivity, specificity and accuracy of results.^{50,51}

A synthetic enzyme substrate is structurally composed of three key components: (i) an enzyme-labile moiety that is specifically recognized and cleaved by the target bacterial enzyme, (ii) a signalogenic unit that generates a detectable signal upon enzymatic cleavage, and (iii) an optional labile spacer designed to prevent non-enzymatic hydrolysis and reduce background signal interference.⁵² Synthetic substrates are commonly classified based on (i) the type of signal they produce (fluorogenic, chromogenic, or luminogenic) and (ii) the nature

of the target enzyme (hydrolytic or non-hydrolytic).⁵² Figure 5 shows the hydrolysis of synthetic enzyme substrates targeting hydrolase.

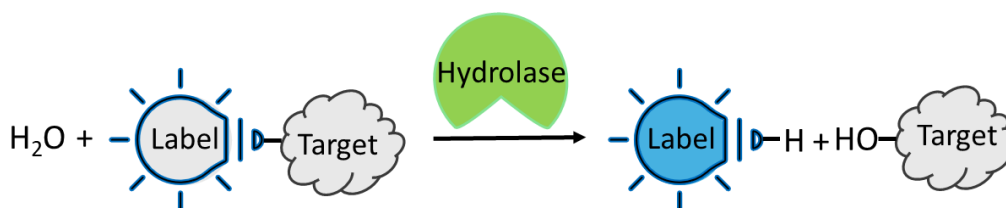


Figure 5: Exemplary hydrolysis of synthetic enzyme substrates.

Fluorogenic substrates generate a fluorescent signal, while chromogenic substrates produce a visible color change upon enzymatic hydrolysis. Both types of substrates function via a similar mechanism in which the enzymatic cleavage of a substrate-signalophore conjugate results in the liberation of a detectable reporter molecule. Among fluorogenic substrates, coumarin derivatives such as 4-methylumbelliferone (4-MU) and 7-amino-4-methylcoumarin (7-AMC) are among the most widely used enzyme substrates. These substrates are favored due to their non-carcinogenic properties and the ease of visualizing fluorescent products under UV light.⁵³ The application of fluorogenic substrates for bacterial enzyme detection was first reported by Dyer in 1970.⁵⁴ For instance, 4-MU is often conjugated to β -D-glucuronide or other sugar moieties for specific detection of bacterial glycosidases or phosphatases. Upon enzymatic cleavage, 4-MU derivatives produce a strong fluorescent signal, which can be quantitatively measured at an emission wavelength of 442 nm following excitation at 366 nm.^{53,54} A notable example is 4-methylumbelliferyl- β -D-glucuronide (MUG), which is widely employed in water quality monitoring to detect β -D-glucuronidase producing *E. coli* strains.⁵⁵ Figure 6 shows the chemical structures of MUG and 4-MU.

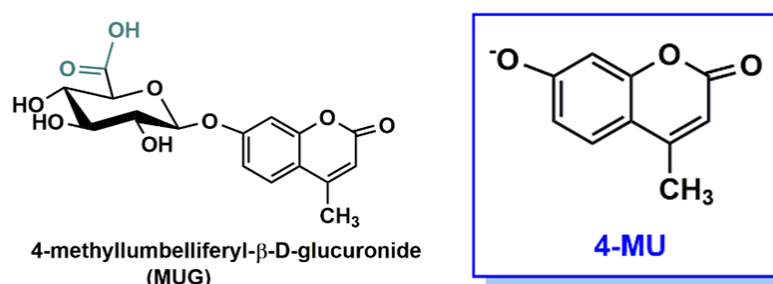


Figure 6: The chemical structures of (left) fluorogenic substrate 4-methylumbelliferyl- β -D-glucuronide, and (right) the released fluorophore 4-methylumbelliferone.

Among the chromogenic substrates, indoxyl-based substrates, initially developed in the 1950s for histochemistry, have since been adapted for use in microbiology.^{34,56} Enzymatic hydrolysis of these substrates releases indoxyl, which oxidizes and dimerizes in the presence of oxygen to form a water-insoluble indigo dye. For example, 5-bromo-4-chloro-3-indoxyl- β -D-galactopyranoside (X-Gal) is used to detect β -galactosidase activity in bacterial colonies.

Upon enzymatic cleavage, X-Gal generates a distinctive blue indigo pigment, facilitating visual detection of colonies on agar plates.⁵⁶ Another chromogenic substrate, 5-bromo-4-chloro-3-indolyl- β -D-glucuronide (X-Gluc) (Figure 7) is widely used to detect β -glucuronidase from *E. coli*. Upon enzymatic cleavage water insoluble indigo dye 5,5'-dibromo-4,4'-dichloro-indigo is formed via dimerization of the released indoxyl group from X-Gluc substrate in the presence of O₂.

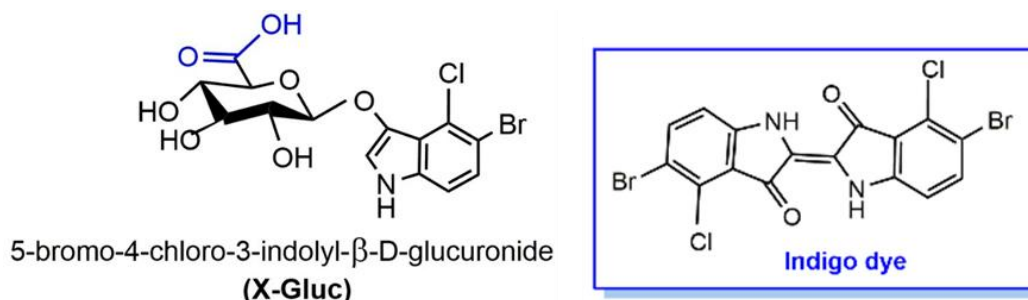


Figure 7: The chemical structures of (left) chromogenic substrate 5-bromo-4-chloro-3-indolyl- β -D-glucuronide, and (right) the released indigo dye.

The color of indigo depends on its environment. It appears red ($\lambda_{\text{max}} = 540 \text{ nm}$) in the vacuum. It appears violet in non-polar solvents like tetrachloromethane ($\lambda_{\text{max}} = 588 \text{ nm}$). Whereas in polar solvents like ethanol ($\lambda_{\text{max}} = 606 \text{ nm}$) and also in the solid state it's blue.^{57,58}

In this work, two kinds of enzyme substrates, i.e., MUG and X-Gluc, are employed for the detection of β -glucuronidase from *E. coli*. Upon enzymatic hydrolysis by β -glucuronidase, both substrates release β -D-glucuronic acid as a common product, while the aglycone moiety differs depending on the specific substrate. The released aglycone is typically a chromogenic (for X-Gluc) and fluorogenic (for MUG) dye, enabling the generation of a visible or fluorescent signal, respectively. So the nature of the dye released varies between substrates, supporting substrate-specific and versatile detection strategies.

2.2.3 Enzyme-responsive polymer-based sensors

Responsive polymers or smart polymers are polymers that can undergo reversible or irreversible changes in their physical properties and/or chemical structures in response to physical, chemical, or biological stimuli from the environment. A range of external stimuli such as pH, temperature, ionic strength, light, electric and magnetic fields, as well as specific analytes or bioactive molecules, have been utilized to study changes in polymer chain conformation, solubility, and self-assembly behavior.⁵⁹ These polymers exist in various physical states, including solutions, hydrogels, self-assembled aggregates, (multilayer) films, and bulk materials. Compared to conventional polymers used in biomedical applications, responsive polymers are especially promising due to their inherent bio-responsive characteristics. As a result, they have been widely proposed for various advanced applications

including diagnostics and biosensing,⁶⁰ drug delivery systems,⁶¹ wound healing, and tissue regeneration.⁵⁹

Among various types of stimuli-responsive polymers, enzyme-responsive polymers exhibit intriguing properties owing to the high specificity and catalytic efficiency of enzymatic reactions. These polymers undergo structural or functional changes upon interaction with specific enzymes, even under physiologically mild conditions. They show reversible or irreversible enzyme-induced changes in their chemical or physical properties. These polymers consist of two core components: (i) an enzyme-sensitive moiety, often a biomolecule such as a peptide, lipid, or polynucleotide that acts as a substrate or substrate mimic, and (ii) a component that modulates covalent or non-covalent interactions, causing macroscopic transitions.⁶² Enzyme-responsive polymers have been developed from polymer hydrogel-based materials to polymer micelles,⁶³ biodegradable capsules,⁶⁴ particles,⁶⁵ polymer conjugates,⁶⁶ and self-immolative polymers.⁶⁷ Both natural and synthetic polymers have been used for enzyme-responsive systems. Due to the exceptional selectivity of the enzymes towards their substrates, their presence allows specific and biologically inspired reactions.⁶⁸ So the enzyme-responsive polymers represent a powerful and highly tunable platform for the detection of bacterial enzymes and associated pathogens.

For bacterial detection, enzyme-degradable polymers have been developed by synthesizing amphiphilic block copolymers composed of hyaluronic acid and either polylactic acid or polycaprolactone, which self-assemble into nanocapsules encapsulating fluorescent or chromogenic dyes.^{69,70} Upon exposure to bacterial enzymes (produced by *S. aureus* and *P. aeruginosa*), degradation of the capsule wall resulted in dye release, thus signaling the presence of the bacterial enzyme and its associated pathogen. Moreover, Ebrahimi et al. reported autonomously reporting hydrogels by conjugating chromogenic or fluorogenic substrates to chitosan films using EDC/NHS chemistry for the rapid and selective detection of bacteria.⁷¹ In the presence of a specific bacterial enzyme, the glycosidic bonds in the enzyme substrates are cleaved off and result in the release of a reporter dye. This strategy was expanded by Jia et al. for the multiplexed bacteria detection and differentiation using chitosan enzyme-responsive hydrogels.^{72,73}

Chitosan as a colorimetric sensor

Chitosan

Chitosan is a natural polysaccharide derived from the deacetylation of chitin, which is the second most abundant polysaccharide available on earth after cellulose.⁷⁴ Figure 8 shows the chemical structures of chitin and chitosan. Chitosan is more suitable for bio-applications than chitin because of its better solubility in water and organic solvents. It consists of randomly

distributed β -(1,4)-linked D-glucosamine and N-acetyl-D-glucosamine units with a degree of polymerization ranging from 500 to 5000. As a functional material, chitosan offers a unique set of characteristics such as biodegradability, biocompatibility, nontoxicity, good adhesion properties due to hydrophilicity, which makes chitosan one of the most popular biopolymers used for fabricating nanocomposites for biosensing applications.⁷⁵

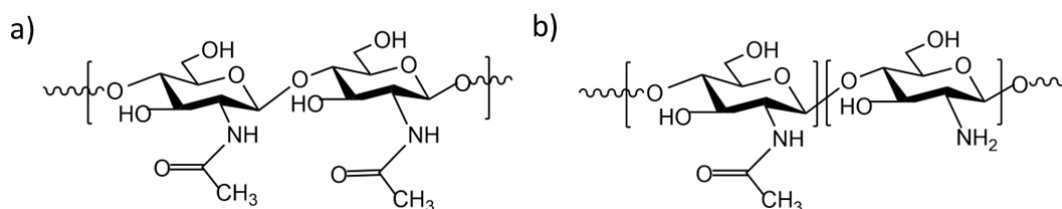


Figure 8. Chemical structure of a) chitin and b) chitosan.

Deacetylation of chitin introduces amino groups in the polymer chain which can be protonated in dilute acidic solutions ($pK_a \sim 6.2$).⁷⁶ The presence of hydroxyl and amino groups on the chitosan backbone allows various chemical modifications, resulting in different applications.^{75,77} Chitosan is used to prepare hydrogels, films, fibers or sponges for various applications. For colorimetric sensing of bacterial enzymes, chitosan-based hydrogels and nanofibers are explored here.

Hydrogel and chitosan hydrogel-based colorimetric sensors

Hydrogels are three-dimensional crosslinked polymer networks capable of retaining large amounts of water within their interstitial spaces without dissolving.⁷⁸ Their swelling in water is driven by hydrophilic groups and osmotic pressure, while crosslinking controls the extent of swelling. The network is formed by chemically or physically crosslinked hydrophilic polymers, ensuring structural stability in aqueous environments. Physical crosslinking involves hydrophobic interactions, hydrogen bonding, or electrostatic interactions, whereas chemical crosslinking involves covalent bond formation. The properties of hydrogels arise from their polymeric building blocks, which enable chemical modifications and allow for tailored network structures with tunable functions, making them suitable for sensing applications.⁷⁹ The response mechanisms of hydrogels play a crucial role in defining their behavior and suitability for sensing applications. These materials react to various stimuli in distinct ways, influencing their sensitivity, selectivity, and effectiveness across different sensing platforms.⁸⁰ One response mechanism involves functionalizing the hydrogel backbone with specific groups that induce color or fluorescence changes upon interaction with target analytes.^{71,73}

Colorimetric chitosan hydrogels for lab-in-a-phone application

To overcome the limitations of conventional diagnostic methods, particularly their reliance on specialized equipment and laboratory settings, Point-of-Care (POC) testing methods have

been developed to enable rapid and accurate bacteria detection.⁸¹ With the widespread availability of mobile phones, smartphone-based biosensors have gained significant attention, paving the way for next-generation POC diagnostics, also referred to as 'lab-in-a-phone' technology.⁸² The integration of this technology with smart interfaces and nanochemistry has shown great promise, especially for use in remote or resource-limited environments.⁸² Paper and paper-based microfluidic devices have been explored as carriers for sensing elements in applications such as healthcare diagnostics, food safety, and environmental monitoring.⁸³ Paper offers advantages such as passive fluid transport via capillary action, compatibility with various chemicals and biomaterials, and reduced issues with air bubbles.⁸⁴ A promising strategy involves integrating a practical bacterial detection method using colorimetric chitosan hydrogels on a paper-based platform to detect the *E. coli* marker enzyme β -glucuronidase, followed by smartphone-based readout and data analysis.

Nanofibers and chitosan nanofibers-based colorimetric sensors

Nanomaterials, characterized by their nanoscale dimensions, have gained considerable interest in sensor and biosensor design due to their distinct properties compared to their bulk counterparts. Depending on their shape and size, these nanoscale structures exhibit unique properties affecting the design and performance of sensing platforms.⁸⁵ Various innovative sensing platforms have been developed using nanomaterial-based components, including nanoparticles,⁸⁶ nanocapsules,⁸⁷ and reporter liposomes for detecting bacterial infections in wound dressings⁸⁷ as well as nanofiber-based sensors.⁸⁸

Electrospinning is widely recognized as an effective, low-cost, and straightforward technique for producing nanofibers for sensing applications.⁸⁵ A typical electrospinning system consists of four key components: a high-voltage power supply, a syringe pump, a metallic spinneret, and a conductive collector. The collector's configuration, such as aluminum foil or a rotating drum, can be chosen based on the desired nanofiber characteristics for specific applications. In this process (Figure 9), the polymer solution is continuously delivered through the spinneret at a controlled flow rate, forming a droplet at the tip. Under the influence of the applied voltage, electrical charges accumulate in the droplet, causing it to elongate into a Taylor cone. Once the voltage surpasses a critical threshold, a fine jet of charged solution is ejected, which is then stretched, elongated, and solidified, generating nanofibers on the collector placed at an optimized distance.⁸⁹

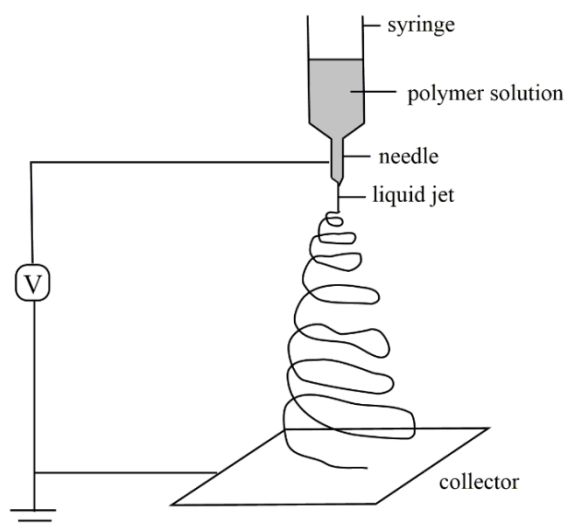


Figure 9: A typical electrospinning set-up.

By controlling the processing parameters (applied voltage, the distance between the spinneret and the collector, and feed rate), solution properties (viscosity, concentration, conductivity, surface tension), and ambient conditions (humidity and temperature), nanofibers with desirable diameters and structures can be obtained, which makes electrospinning a versatile and effective method for producing engineered fibers with several attractive properties for sensing applications. Electrospun nanofibers are characterized by their inherently high surface-area-to-volume ratio and significant porosity, both of which are critical for enhancing biosensor performance.⁹⁰ The extensive surface area allows for the effective immobilization of a large amount of biomolecules and facilitates strong interactions with target analytes, thereby improving sensor sensitivity. In addition, their porous structure enhances analyte accessibility and reduces resistance to mass transport, promoting efficient diffusion through the sensing layer.⁹¹

Among the different types of electrospun nanofibers, those derived from chitosan have been extensively studied for their antibacterial and biomedical applications.⁹² Additionally, electrospun fibers made from polymers such as poly(ethylene oxide), polyurethane, and poly(vinyl alcohol-co-ethylene) have been employed as colorimetric sensors for bacterial detection, particularly targeting non-pathogenic *E. coli*.^{93,94,95} Chitosan-based electrospun nanofibers incorporating chromogenic or fluorogenic substrates take advantage of both chitosan's intrinsic properties and the enhanced surface area of the nanofiber structure, offering improved sensitivity for detecting pathogenic *E. coli* compared to traditional hydrogel systems.

2.3 Antimicrobial photodynamic therapy for bacteria eradication

2.3.1 Antimicrobial resistance

Antimicrobial resistance (AMR) poses substantial burden on public health systems, as already mentioned in Chapter 1. It arises when microorganisms such as bacteria, viruses, fungi, and parasites develop the ability to resist the effects of drugs designed to eliminate them. This resistance renders antibiotics and other antimicrobial agents ineffective, making infections harder or sometimes impossible to treat resulting in severe health complications, long-term disability, and death. This growing resistance problem stems largely from the excessive and inappropriate use of antibiotics, coupled with a decline in the development of new antimicrobial drugs.⁹⁶

A significant number of drug-resistant infections encountered in clinical settings are linked to a particular group of pathogens collectively referred to as the 'ESKAPE' organisms. This group includes *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and various *Enterobacteriales* species.⁹⁷ These pathogens are known for their ability to adapt and persist within healthcare environments. They possess a wide array of innate and acquired resistance mechanisms, which have allowed them to emerge as leading contributors to antimicrobial-resistant infections over time.⁹⁷ The mechanisms of AMR can be classified into four categories: (i) reduced drug uptake, (ii) alteration of the drug target, (iii) enzymatic inactivation of the drug, and (iv) drug efflux from the cell. Due to their structural features, particularly the presence of an outer membrane, Gram-negative bacteria are capable of employing all four resistance strategies. Whereas Gram-positive bacteria are less likely to use limiting drug uptake (the lipopolysaccharide in the outer membrane is absent) and drug efflux mechanisms.⁹⁸

2.3.2 Bacterial infections in Cystic Fibrosis lung

Cystic fibrosis (CF) is a genetic disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which encodes a chloride and bicarbonate ion transport channel, essential for maintaining osmotic balance across epithelial surfaces.⁹⁹ Dysfunction of this channel disrupts ion transport across epithelia, leading to thickened airway surface liquid, impaired mucociliary clearance, and the buildup of dehydrated, viscous mucus.¹⁰⁰ This compromises immune defense in CF, increasing susceptibility to both acute and chronic respiratory infections. Over time, the interplay between bacterial virulence and the host inflammatory response causes progressive lung damage,

often culminating in respiratory failure and the need for lung transplantation.⁹⁹ The CF environment favors persistent colonization by opportunistic pathogens, most notably *P. aeruginosa* and *S. aureus*, which are major contributors to chronic lung infections in CF patients.

P. aeruginosa is a particularly resilient and predominant pathogen in the CF lung. It readily adapts to hypoxic conditions through genetic mutations and phenotypic changes, such as transitioning to a mucoid form characterized by overproduction of alginate. This form supports biofilm formation.¹⁰¹ These biofilms, along with *P. aeruginosa*'s metabolic flexibility and capacity for mutation, make eradication nearly impossible once chronic infection sets in. Furthermore, frequent antibiotic treatments create selective pressure that drives the emergence of resistant strains and small-colony variants, which further enhance biofilm formation and persistence.¹⁰² *S. aureus* is also frequently isolated from CF airways, particularly in children, and contributes to inflammation and early lung damage. Co-infection of *P. aeruginosa* and *S. aureus* is common in CF patients, which may exacerbate disease severity due to complex interspecies interactions. Other organisms, including *Candida albicans* and *Aspergillus fumigatus*, can also be present, forming polymicrobial communities that complicate treatment.¹⁰³

The nutritional composition of CF mucus is known to be composed of amino acids, mucin, DNA, lipids, and micronutrients, including metal ions, which support the growth of these pathogens.¹⁰⁴ To study their physiology under CF-like conditions, researchers use specialized in vitro models such as Artificial Sputum Medium (ASM) and Synthetic Cystic Fibrosis Medium (SCFM). These media replicate the chemical environment of CF sputum and allow investigation of microbial behavior, including biofilm formation and antibiotic resistance, under conditions that closely mimic those in the CF lung.¹⁰⁴

The eradication of chronic CF infections and biofilms remains a major challenge due to their intrinsic resistance mechanism. Conventional antibiotic therapy often fails, as biofilm-embedded bacteria exhibit up to 1,000 times greater resistance than their planktonic counterparts.¹⁰⁵ The pulmonary physiological impairments of CF airways, such as the presence of viscous mucus, lowered pH, and osmotic and oxidative stress, impair the efficacy of antibiotics by reducing their stability and diffusion, often resulting in sub-inhibitory concentrations within the sputum.^{106,107} Moreover, bacteria residing in biofilms exhibit resistance to antibiotics at levels 100-1000 times higher than their planktonic counterparts.¹⁰⁸ Several strategies have been explored to address this issue, including antimicrobial peptides,¹⁰⁹ bacteriophage therapy,¹¹⁰ and quorum-sensing inhibitors.¹¹¹ Despite these advances, prolonged and repeated antibiotic treatments promote the emergence of multidrug-

resistant (MDR) organisms, limiting the treatment options for patients with advanced disease stages.¹¹² The emergence of MDR is increasingly reported in CF populations,¹¹³ further underscoring the urgency for alternative antimicrobial strategies. Among alternative antimicrobial strategies, bacteriophage therapy, which employs viruses that selectively target bacteria, has shown its ability to reduce bacterial burden and disrupt biofilms in CF-related infections. However, further large-scale clinical trials are necessary to fully assess its safety and therapeutic efficacy.¹¹⁴

2.3.3 Antimicrobial photodynamic therapy

Antimicrobial photodynamic therapy (aPDT) has emerged as a potential alternative treatment for resistant bacterial infections, including those in CF. Several studies have reported the efficacy of aPDT in inactivating both *P. aeruginosa* and *S. aureus*, including antibiotic-resistant strains and biofilm-embedded populations,¹¹⁵ thereby enhancing the susceptibility of these bacteria to conventional antibiotics and host immune mechanisms.¹¹⁶

aPDT involves the administration of photosensitizers (PS) which, upon light activation, generate reactive oxygen species that disrupt bacterial membranes and biofilms. In a typical aPDT process (Figure 10), upon absorbing a photon of light, the PS transitions from ground state (S_0) to an excited singlet state (S_1), which is highly reactive but short-lived. From this excited state, the PS may return to its ground state either by releasing energy as fluorescence or it can undergo intersystem crossing (ISC) to a more stable, longer-lived triplet excited state (T_1). Once in the triplet state, the PS can follow two main photochemical pathways, Type I electron transfer and Type II energy transfer. In the Type I pathway, the triplet PS accepts an electron from a donor molecule (D) present in its vicinity, which induces an electron transfer, producing a radical cation on the donor ($D^{\bullet+}$) and a radical anion on PS ($PS^{\bullet-}$). In the presence of oxygen, the superoxide anion radical ($O_2^{\bullet-}$) is generated, which after subsequent reduction, leads to the generation of more cytotoxic reactive oxygen species (ROS), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\bullet}$). In the Type II pathway, energy is directly transferred from the triplet PS to ground-state molecular oxygen ($^3O_2$), producing singlet oxygen ($^1O_2$), a highly cytotoxic and short-lived species. Among all produced ROS, $HO^{\bullet}$, and $^1O_2$ are particularly the most reactive and cytotoxic.¹¹⁷

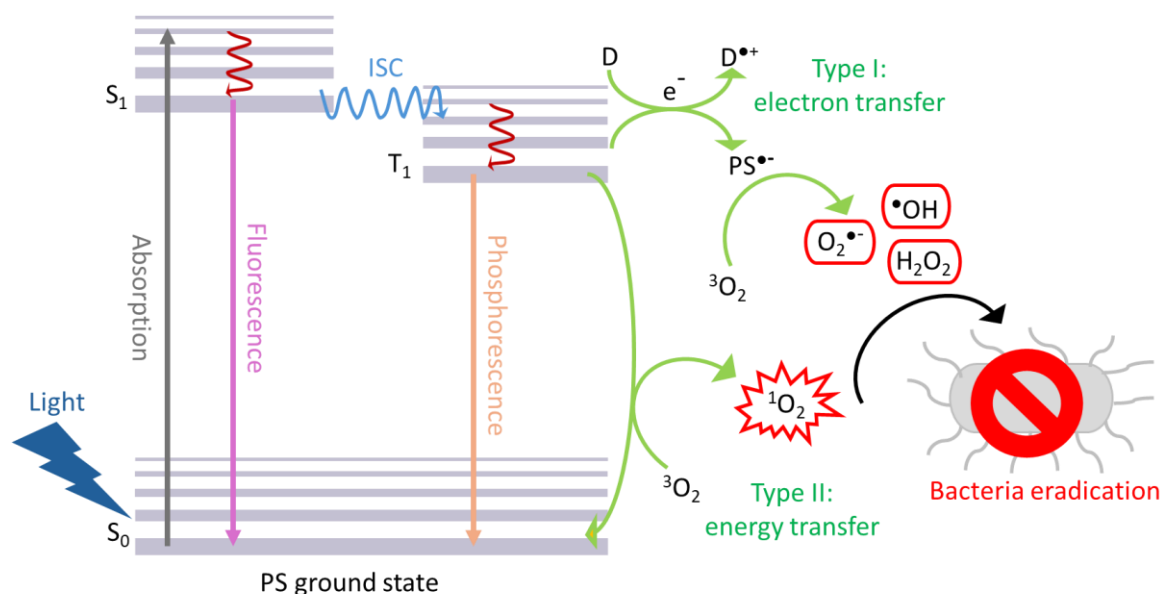


Figure 10: Schematic illustration of the antimicrobial photodynamic therapy mechanisms.

Notably, a single PS molecule can give rise to thousands of $^1\text{O}_2$ molecules, influenced by factors such as its singlet oxygen quantum yield, the surrounding environment, and the respective occurrence of Type I and Type II processes.^{118,119} The effectiveness of aPDT depends on the type of PS used, the wavelength of light, and the oxygen availability within the biological medium and biofilms.^{118,120} In addition to being effective against a broad range of microorganisms, aPDT also has several advantages, including high selectivity with rapid action as compared to conventional antimicrobial drugs, efficacy to eliminate biofilm, and is relatively cost-effective.^{121,122} For many common MDR bacteria, aPDT has already been proven to be a strategy to avoid the build-up of resistance, as the killing of bacteria is based on the action of reactive oxygen species, which simultaneously addresses multiple targets in the bacterial cell and hence impede the resistance build-up.¹¹⁷

PS used in aPDT

Since the early 1990s, many different systems have been investigated for aPDT, including novel photosensitizers over photoactive surfaces and different kinds of nanoparticles, fibers, and nanocarriers of various origins.^{117,120,123} PS's used in aPDT can be broadly classified into natural and synthetic compounds. Natural PS's are derived from biological sources and include curcumin, hypericin, riboflavin, and phycocyanin. Whereas synthetic PS include porphyrins, chlorins, and various organic dyes, including methylene blue and toluidine blue.¹²⁴ Natural PS's are known for their low toxicity, high selectivity, better safety profiles than synthetic options, and ease of accessibility. However, they also present challenges, including rapid clearance from the bloodstream and limited accumulation at target sites such as tumors or infection zones. Additionally, the extraction of active compounds from plant sources is often time-consuming and labor-intensive due to variations in chemical composition influenced by

geographical and environmental factors, which may compromise their reproducibility and effectiveness in photodynamic applications. To address these limitations, researchers have developed nanoparticle-based systems by combining PS's with natural cell membranes to improve delivery and targeting.¹²⁵ In addition, various metal-based PS have also been investigated for their potential in aPDT. In 2022 de Siqueira et al. reviewed the applications of phthalocyanines complexed with metals such as aluminium, cobalt, gallium, silicon and zinc in different nanocarriers, such as liposomes, for targeting bacteria, fungi, viruses and protozoans.¹²⁶ In addition, ruthenium (II) complexes (Ru(II)) have been extensively studied for PDT effects against fatal cancers, and some lead complexes undergo phase II clinical studies for PDT treatment of bladder cancer.^{127,128} Despite several developments, challenges with the solubility and stability in aqueous media remain to be improved to achieve optimal performance in medical applications. To overcome these challenges, aerosolized and nebulized antibiotic delivery has been employed to enhance local drug concentrations in the lungs, with up to 100-fold higher concentrations than systemic administration.¹²⁹ Nonetheless, many known PS used for aPDT show only limited biocompatibility, limited solubility in aqueous or biological media, dark toxicity and reduced photoactivity in the aggregated state, which leads to difficulties in applications.^{130,131}

Nanocarriers for PS delivery

One effective strategy to address these limitations is adapted from the drug delivery of water-insoluble drugs. Several polymer-based nanocarriers have been designed to enhance the stability, adaptability, and durability of PS delivery systems. The established carriers for encapsulation include polymeric micelles, polymersomes, liposomes, and a variety of nanoparticles.^{132,133} An important feature for such systems is their ability to circulate in the body without releasing their payload prematurely and to release the cargo on demand at the site of infection. Amphiphilic block copolymers, composed of covalently linked hydrophilic and hydrophobic segments, offer a straightforward strategy for forming self-assembled and kinetically stable nanostructures.¹¹⁷ Poly(ethylene glycol) (PEG) is frequently incorporated as the hydrophilic block due to its biocompatibility and its 'stealth' properties, which help prolong systemic circulation by minimizing protein adsorption.¹³⁴ Polylactic acid (PLA), often used as the hydrophobic block, is enzyme-labile and enables on-demand PS release. PLA can be selectively degraded by proteinases, such as those secreted in high amounts by *P. aeruginosa* at infection sites.¹³⁵ PLA-based block copolymers have previously demonstrated the enzyme-triggered release of encapsulated signaling molecules upon exposure to proteolytic enzymes like proteinase K.^{70,136} This strategy was adapted for the encapsulation of potent ruthenium-based PS's in PEG-*b*-PLA_x, enabling the targeted and effective eradication of clinically relevant bacterial species using aPDT.

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3 Chitosan-based hydrogel sensing substrate

This Chapter outlines the detection of the *E. coli* enzyme β -Gus using a chitosan-based sensing hydrogel-coated paper sensor, with RGB analysis of the data captured via a smartphone camera. Part of the work presented in this chapter has been published in Biosensors.ⁱ The experiments and formal analysis described herein were performed by the author.

3.1 Introduction and objectives

Bacterial pathogens pose a significant global threat, particularly in relation to food and water safety as well as public health. Regardless of geographical location or socioeconomic conditions, the absence or inadequacy of rapid, reliable, and cost-effective diagnostic tools for detecting these bacteria can increase the risk of pathogen transmission which can lead to severe or even life-threatening diseases.¹ To address these challenges, there is urgent need for the development of new, low-cost, and user-friendly point-of-care bacterial detection methods. Such diagnostics methods could help overcome the shortcomings of traditional methods, especially for certain application settings.

One promising strategy involves the use of colorimetric sensors based on enzyme-catalyzed reactions which allow the autonomous and visible detection of bacteria.^{2,3,4,5} Among these, chitosan-based sensing hydrogels have been extensively studied for their rapid, selective, and visually detectable response, making them highly suitable for effective bacterial identification.^{36,3,6} Chitosan hydrogels functionalized with various fluorogenic or chromogenic substrates have previously demonstrated the ability to rapidly, selectively, and sensitively detect and differentiate between different bacterial strains. In several instances, these hydrogels achieved enzyme LOD below 1 nM within 60 min observation time.^{4,7}

With the widespread global availability of mobile phones, smartphone-based biosensors have gained significant attention, driving advancements in POC sensing and biosensing technologies. These developments have paved the way for next-generation applications, also referred to as "lab-in-a-phone technology" enabling innovative approaches

ⁱ Kaur, K.; Chelangat, W.; Druzhinin, S.I.; Karuri, N.W.; Müller, M.; Schönherr, H. Quantitative *E. coli* enzyme detection in reporter hydrogel-coated paper using a smartphone camera. *Biosensors (Basel)* **2021**, *11*(1), 25.

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to decentralized diagnostics.^{8,9} For instance, Kim et al. reported on a smartphone-based sensor for determining blood hematocrit from 10% to 65% with a LOD of 0.1%. They determined the hematocrit concentration from the red, green and blue components of images captured under white light illumination in a microfluidic device using a smartphone camera.^{10,11} Liu et al.¹² proposed a smartphone sensor to measure concentrations of the antibiotic streptomycin in different food products. The detection of changes in absorbance ratio due to streptomycin binding to aptamer conjugated Au nanoparticles was realized by a smartphone camera.^{10,12} Likewise the concentrations of BSA proteins, catalase enzymes, and carbohydrates were determined using an analysis of the average brightness of smartphone acquired images, which were obtained by converting the image pixels to HSV (hue, saturation, value) color space.^{10,13}

The integration of lab-in-a phone technology with smart interfaces and nanochemistry has shown great potential, particularly for bioanalytical applications in remote and resource-limited environments.^{8,14} Paper and paper-based microfluidic devices have been developed as platforms for reporting units in fields such as healthcare diagnostics, food safety, and environmental monitoring.¹⁵ Paper eliminates the need of pumps, since the wicking mechanism allows for passive transport via capillary action. Moreover, paper is also compatible with a variety of chemicals and biomaterials (biological inertness) and eliminates problems associated with bubbles.^{16,17}

This chapter introduces a method for bacterial detection integrating a paper-based carrier platform with readout via a standard smartphone camera followed by RGB analysis (Figure 1). The approach leverages the color intensity variations of a released dye, modulated by different enzyme concentrations proportional to bacterial presence. For instance, higher bacterial concentrations yield more enzyme production, resulting in increased color intensity. Images captured under various experimental conditions form a comprehensive data library, facilitating rapid, field-applicable bacterial detection. This colorimetric detection technique, demonstrated for *E. coli* holds potential for adaptation to other sensing systems that rely on colorimetric changes.

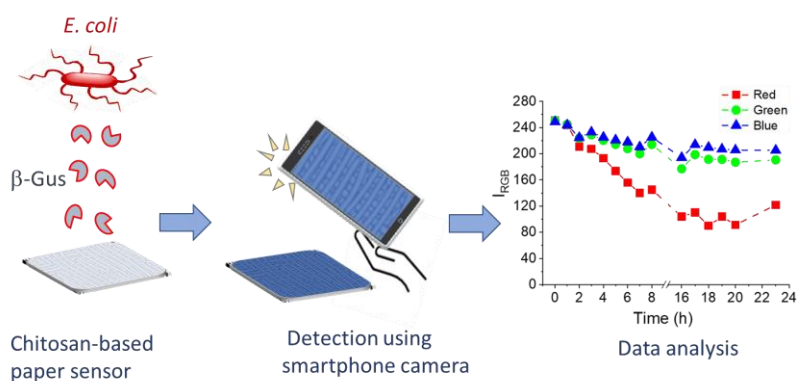


Figure 1. Schematic of the paper-based detection of enzyme β -Gus using the smartphone camera.

3.2 Results and discussion

3.2.1 Characterization and functionalization of chitosan-modified paper

Filter paper with pore size 12 - 15 μm was modified with chitosan by repeated casting from 1% acetic acid solution, followed by drying. The presence of the chitosan on filter paper was confirmed by X-ray photoelectron spectroscopy (XPS). The XPS survey spectra (Figure 2a) showed the presence of carbon, nitrogen and oxygen. The detailed XPS element scans in Figure 2b-d show the corresponding C1s, O1s and N1s signals. After coating the paper with chitosan, the characteristic N1s signal indicates the presence of chitosan in the samples.

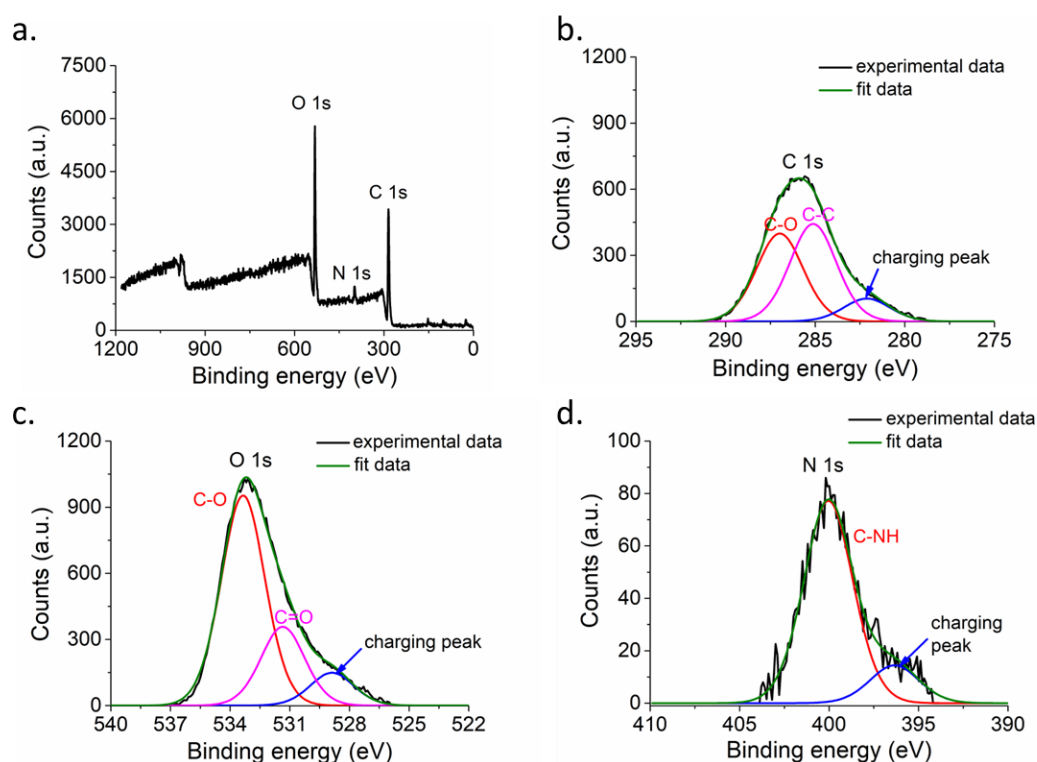


Figure 2. XPS survey scan of chitosan-modified paper (prepared from 1.5 wt% chitosan solution in acetic acid). The high-resolution spectra of 1.5 wt% chitosan deposited on paper substrate for (b) C1s, (c) O1s and (d) N1s.

In all spectra a significant contribution of uncompensated charging was observed, which was corrected for by fitting a separated peak. The C1s signal (Figure 2b) was deconvoluted into two peaks at 286.9 eV and 285.1 eV, which are attributed to carbon species bonded to oxygen (C-O) and aliphatic carbon (C-C), respectively. The oxygen signal (Figure 2c) contained contributions of oxygen as C-O at 533.3 eV and C=O at 531.5 eV. Finally, the corresponding N1s signal was attributed to one N species –C-NH, centered at 400.0 eV (Figure 2d).

The wet chemical functionalization of the chitosan coated paper was carried out using established protocols that rely on the activation of carboxylic acid groups of the chromogenic X-Gluc by EDC-NHS chemistry followed by conjugation to the primary amino groups of the chitosan coating.^{4,7} The schematics is shown in Figure 3.

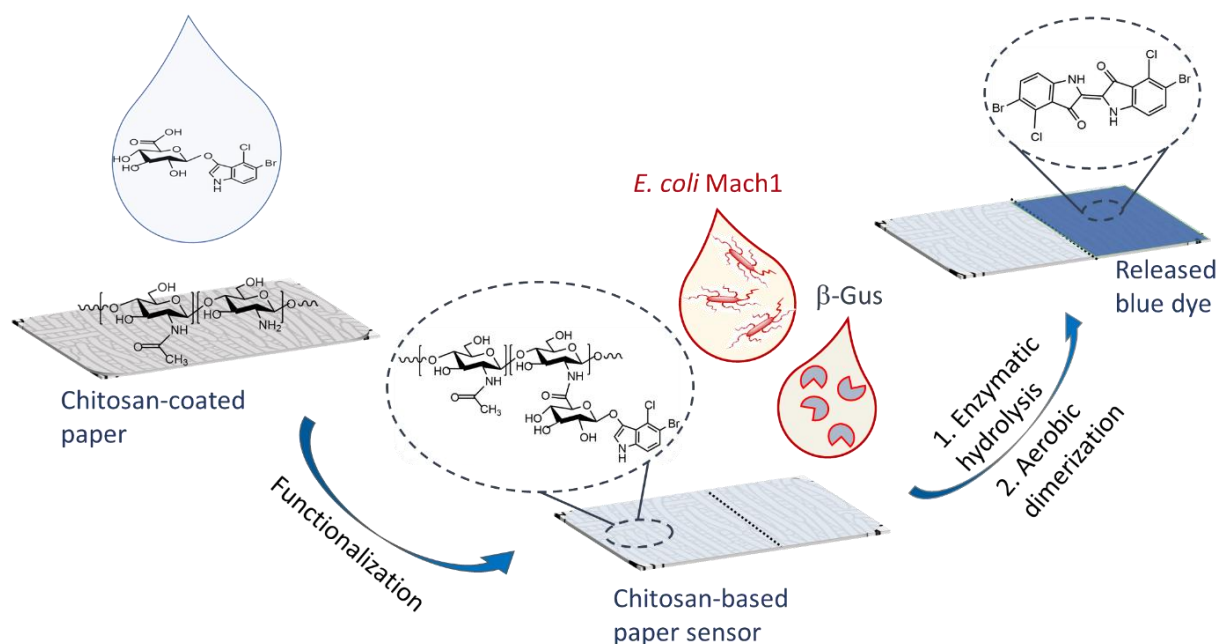


Figure 3. Schematic of paper-based detection of the bacterial enzyme β -Gus. An ultrathin cellulose fiber sheet (with 0.16 mm thickness) serves as the substrate for chitosan functionalization with chromogenic substrate, enabling colorimetric sensing. The enzyme-catalyzed reaction of 5-bromo-4-chloro-3-indolyl- β -D-glucuronide (X-Gluc) in the presence of oxygen yields a deep blue, water-insoluble indigo derivative, which can be analyzed by adequate analysis of smartphone camera photographs.

The formation of amide bonds between X-Gluc and chitosan can be concluded from the attenuated total internal reflection ATR-FTIR spectra acquired prior to and after the reaction (Figure 4).

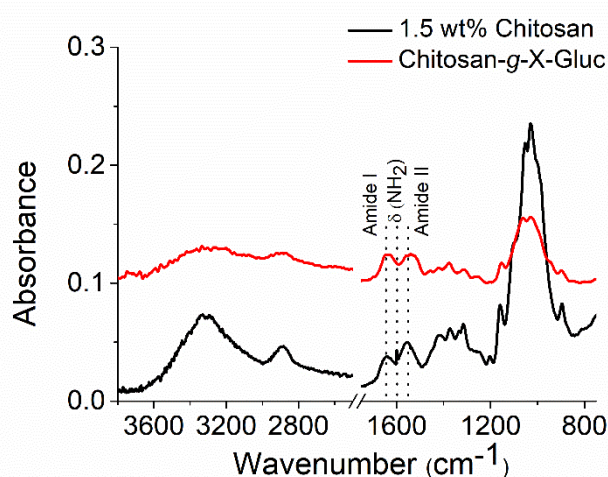


Figure 4. ATR-FTIR spectra of paper modified with chitosan (sample coated twice using 1.5 wt% chitosan solution in 1% acetic acid, black trace) and chitosan functionalized with the chromogenic substrate X-Gluc (sample coated twice using 1.5 wt% chitosan solution in 1% acetic acid, red trace).

The disappearance of the deformation vibration centered at 1599 cm^{-1} upon reaction and the altered contributions of amide I and amide II bands is consistent with the successful

attachment of X-Gluc (Table 1). The ATR-FTIR spectra were in agreement with previously published data which showed that the chromogenic substrate X-Gluc was successfully immobilized on the chitosan layer coating of the paper sensors. After modification with X-Gluc, the primary amine peak in chitosan chain at 1599 cm^{-1} disappeared, while the amide I and II vibrations at 1641 cm^{-1} and 1557 cm^{-1} , respectively, became stronger.

Table 1. Assignment of most prominent bands observed in ATR-FTIR spectra for chitosan films on paper (2 coatings, 1.5 wt %) and chitosan on paper (2 coatings, 1.5 wt %) grafted with chromogenic substrate X-Gluc.

Vibrations and assignments	Chitosan		Chitosan-g-X-Gluc
	data	ref ^{18,19}	
ν_s (O-H, N-H)	3334	3435-3330	3335
ν (C-H) _{aromatic}	-	-	3074
ν (C=O) amide I	1645	1620-1655	1641
δ_b (NH ₂)	1599	1590-1610	-
ν (N-H) amide II	1553	1550-1565	1557
δ_b (C-H)	1375	1375-1382	1376
ν_s (C-O-C)	1053	1065-993	1065
ν_s (C-O-C bridge)	896	890-900	898

3.2.2 Enzymatic reactions of chitosan-g-X-Gluc

For studying the enzymatic reaction systematically, 100 μL of the sterilized β -Gus in PBS (pH 7.4) or in LB (pH 7) were diluted to a designated concentration of the enzyme deposited on the functionalized paper in a custom-made sample holder that was placed in a black box. Photographs were taken at different time intervals with a conventional mobile phone camera under white light illumination. Depending on the concentration and reaction time, the characteristic blue color of the indigo-like dimerized dye was observed. Both in PBS (Figure 5a) and LB medium (Figure 5b) the neat enzyme solutions caused a characteristic blue color, which did not leach from the hydrogel coating.

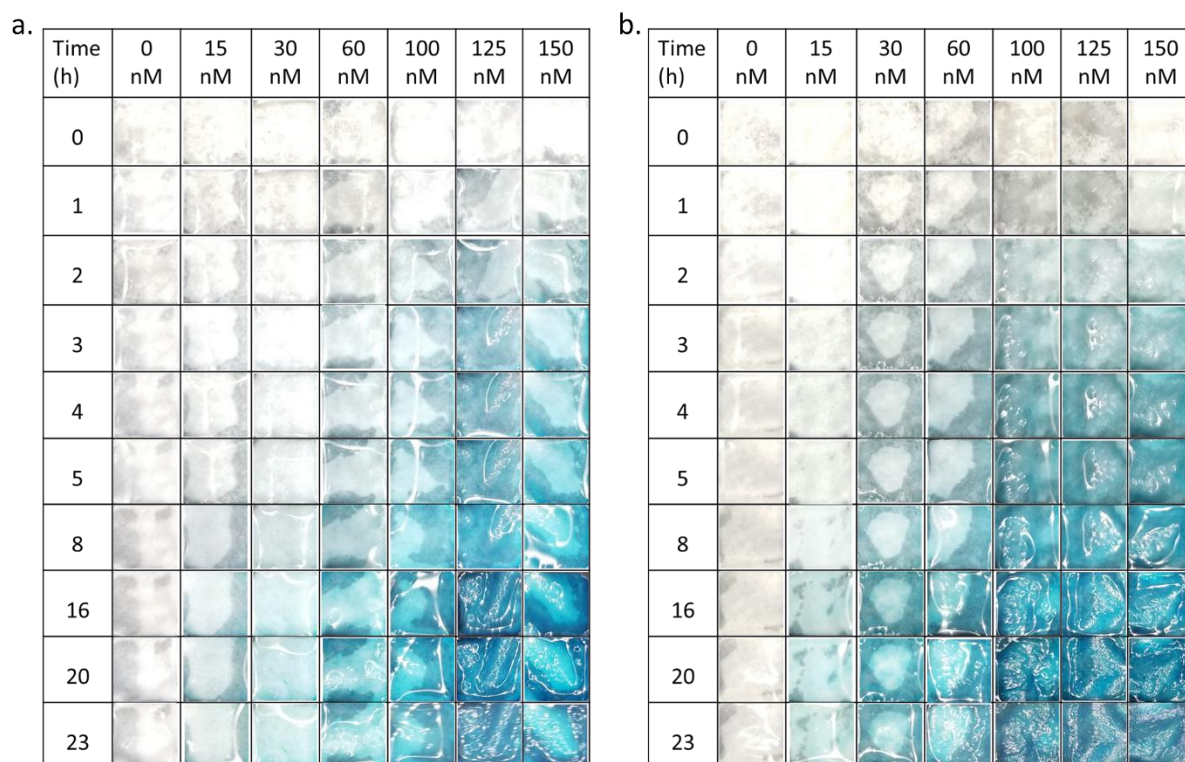


Figure 5. Photographs of visible color change of chitosan-g-X-Gluc coated paper after incubation with different specified β -Gus concentrations (a) in PBS and (b) in LB.

The presence of the substrate residuals was also observed in enzymatic reaction under various conditions. With increasing reaction time, the color intensity progressively increased. In both PBS and LB, the blue color became visible to the naked eye after 2 h of incubation for enzyme concentrations of 60 nM and above. So the LOD of bare eye detection of β -Gus detection using paper sensing substrate was 60 nM when observed after 2 h.

Similarly, the paper sensors were exposed to 100 μ L of a suspension of nonpathogenic *E. coli* Mach1. The desired dilutions of bacteria suspension were prepared in LB, applied to the paper and analyzed by photography after specified reaction times at 20 °C (Figure 6). Depending on the dilution factor of the bacterial suspension and the reaction time, the characteristic blue color of indigo dye was observed.

	LB control	Dilution factor of <i>E. coli</i> MACH-1 suspension (x times)							<i>E. coli</i> MACH-1 ONC in LB
		200	100	50	40	20	10	5	
CFU/mL→	0	1.5 x 10 ⁶	3.0 x 10 ⁶	6.0 x 10 ⁶	7.5 x 10 ⁶	1.5 x 10 ⁷	3.0 x 10 ⁷	6.0 x 10 ⁷	3.0 x 10 ⁸
Time (h)↓									
0									
8									
17									
22									
25									
29									
33									
40									
46									

Figure 6. Photographs of visible color change of chitosan-*g*-X-Gluc coated paper after incubation with different specified *E. coli* Mach1 suspension concentrations in LB.

The appearance of blue color was first observed after 8 h of incubation for samples exposed to a bacterial suspension concentration of 3.0×10^8 CFU/mL. Longer incubation times were necessary to detect the blue color in samples with lower bacterial concentrations. No color change was observed for bacterial concentrations of 6.0×10^6 CFU/mL or lower. So the LOD of the bare eye detection of β -Gus from *E. coli* suspension was 3.0×10^8 CFU/mL observed after 8 h. Additionally, prolonged incubation led to sample drying in some cases, resulting in the diminished intensity of the blue coloration. Qualitatively similar observation in *E. coli* suspension indicates the presence of the enzyme also in the LB medium (Figure 6), in agreement with previously published work.⁷ Bare eye visual detection possesses inherently lower LOD values⁶ and is also hampered by possible observer bias as well as color weakness and blindness issues of the observer.

RGB analysis

In the experiments, the enzymatic cleavage of chitosan-*g*-X-Gluc by β -Gus leads to the primary product, 5-bromo-4-chloro-3-hydroxyindole, and finally by aerobic oxidation to an insoluble final product 5,5'-dibromo-4,4'-dichloro-indigo. The amount of reaction product at time *t* is estimated by a Samsung SM-G950W color digital image sensor having three primary red–green–blue (*RGB*) bands with maxima at 470, 540 and 620 nm, respectively²⁰. The final

reaction product, i.e. the blue pigment possesses strong absorption in the *R*-channel and practically no absorption both in the *G*-channel and in the *B*-channel, as shown in Figure 7.

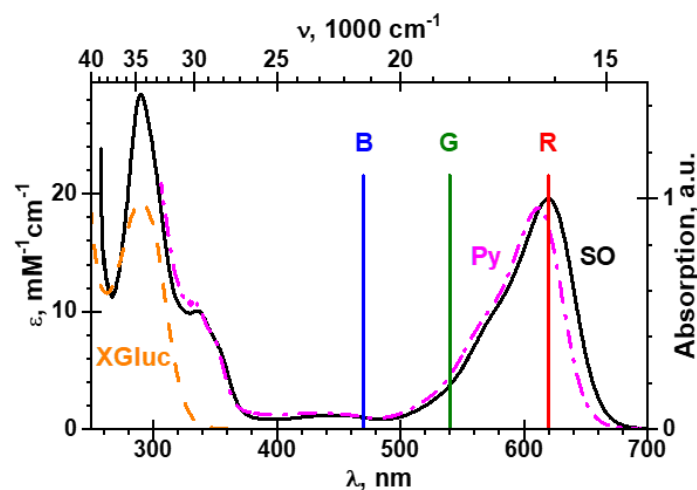


Figure 7. Absorption spectra of chromogenic substrate S, X-Gluc (X-Gluc, dash, orange; for chemical structure, see Figure 1), in PBS, extinction coefficient (ϵ) of indigo in DMSO (SO, solid, black) and pyridine (Py, dash dot, magenta) at 25 °C. The maxima of indigo in DMSO are located as $\epsilon_{\max} = 19600$ and $28500 \text{ M}^{-1}\text{cm}^{-1}$ at 619.6 and 289.0 nm, respectively, while the maximum of indigo in pyridine is found as $\epsilon_{\max} = 18800 \text{ M}^{-1}\text{cm}^{-1}$ at 611.7 nm. The centers of red–green–blue (RGB) bands²⁰ are indicated with the vertical lines.

Hence the *R*-channel is optimal for the detection of the final product. The experimental intensities I_{RGB} were estimated as integral intensities in the *RGB*-channels, followed by the splitting of the measured images with ImageJ software. To investigate the effect of enzyme concentration on RGB intensities, an incubation time of 8 h was chosen, as this was the time point at which color development was first observed at the lowest enzyme concentration (15 nM) (Figure 8).

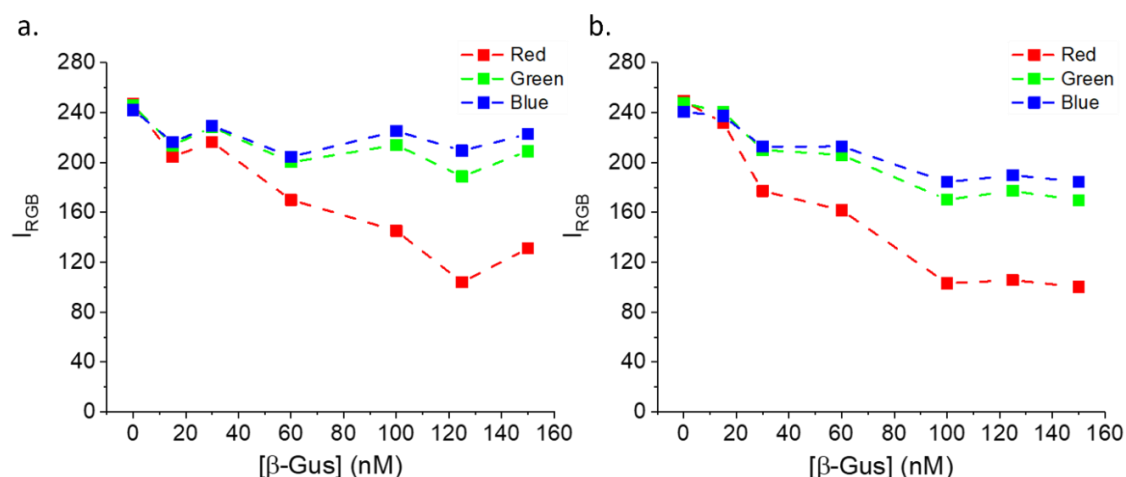


Figure 8. RGB analysis of chitosan-*g*-X-Gluc coated paper after 8h incubation with different β -Gus concentrations (a) in PBS and (b) in LB.

Enzymatic degradation resulted in a reduction of the red channel color index in the images as enzyme concentration increased. While the green and blue channel indices exhibited a slight

decrease. When comparing PBS and LB, a reduction in the red channel was first observed at an enzyme concentration of 60 nM for PBS and 30 nM for enzyme solutions prepared in LB medium.

Additionally, RGB intensities were plotted for a 100 nM enzyme concentration to study the effect of incubation time (Figure 9).

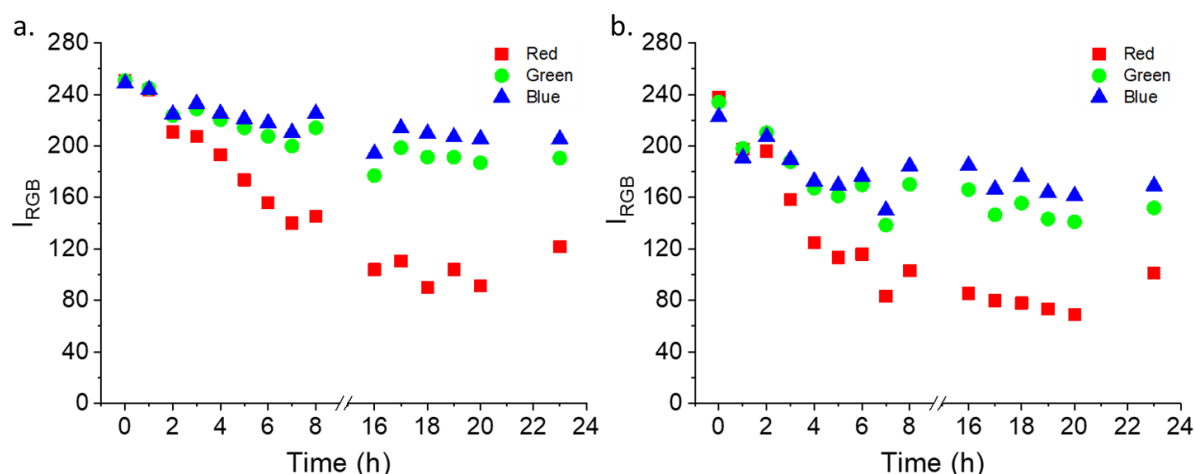


Figure 9. RGB analysis of chitosan-g-X-Gluc coated paper incubated with 100 nM β -Gus prepared in (a) PBS and (b) LB studied at different time intervals.

A reduction in the red channel intensity was also observed with increased incubation time, with a slightly more reduction noted in LB medium compared to PBS.

Figure 10 presents the RGB analysis for samples incubated with varying concentrations of bacterial suspensions over different time intervals. A more pronounced reduction in the R-channel was observed at higher bacterial concentrations (Figure 10a), indicating more product formation through the enzymatic reaction. Data fluctuations were observed at extended incubation times, likely attributable to sample desiccation. Lower bacterial concentrations showed only slight reductions in color intensity.

Figure 10b illustrates the effect on the green channel. As product formation is primarily indicated by changes in the red channel, the fluctuations in green channel intensity at higher bacteria concentrations may be due to the absorption of different components within the complex bacterial suspension. In contrast, the blue channel (Figure 10c), displayed relatively consistent color intensity with minor fluctuations across various bacterial concentrations. So the images captured under various experimental conditions provide the foundation to form a comprehensive data library, facilitating rapid, field-applicable bacterial detection.

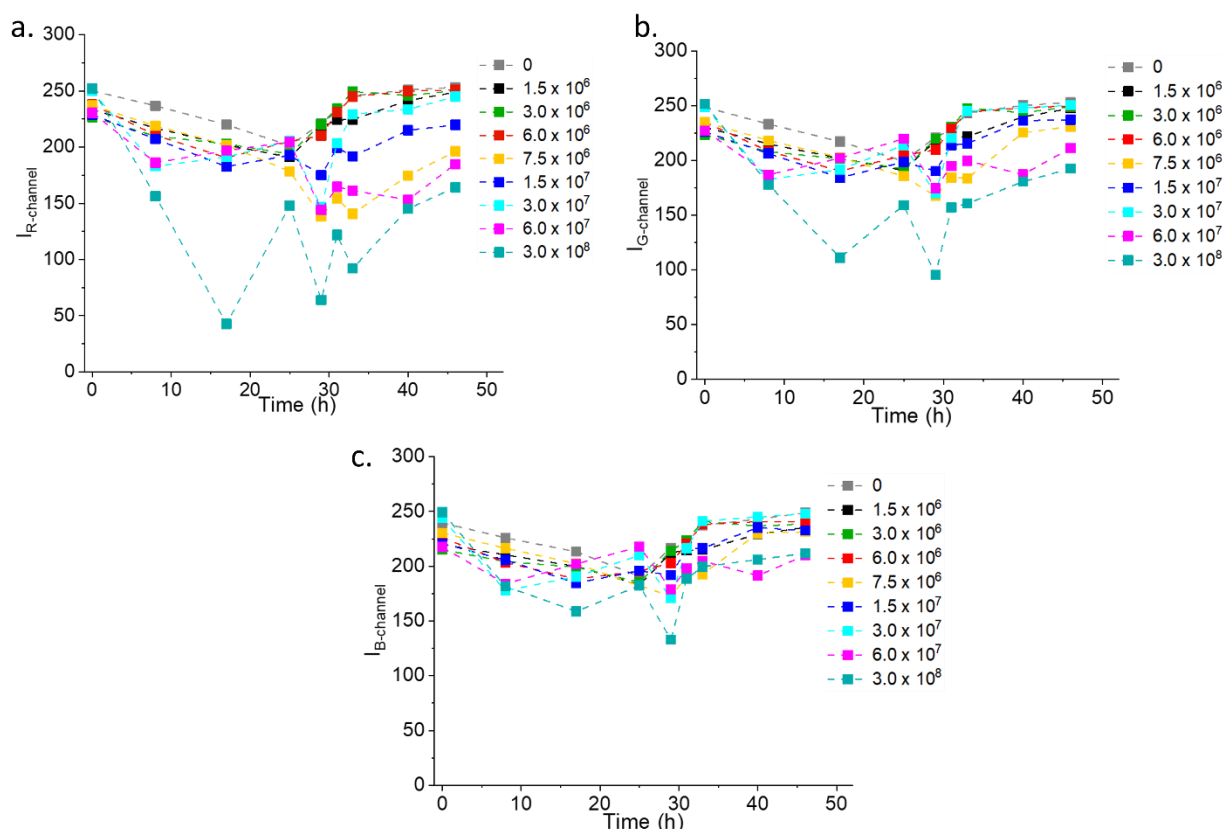


Figure 10. RGB analysis of chitosan-g-X-Gluc coated paper incubated with different concentrations of bacteria suspension (in CFU/mL) with (a) R-channel, (b) G-channel, and (c) B-channel plotted at different time intervals.

These data were compared favorably to literature data. Detection of β -Gus and β -galactosidase by formation of indigo-derivatives in transparent chitosan film was noted to possess LOD values of 3-5 nM for detection with an absorption spectrometer and around 30-60 nM for bare eye visual detection.⁶ It was shown that with reporter-hydrogel coated glass 40 nM of β -Gus in solution can be detected within 5 min employing high quality research-grade absorption spectrometers.²¹ Here it is reported that for the same biochemical system, simple smartphone-based paper biosensors are also shown to provide similar LOD of 60 nM for bare eye detection of β -Gus.⁶ For *E. coli* bacterial suspension with a concentration of 3.0×10^8 CFU/mL longer incubation time of 8 h is required for bare eye detection of β -Gus.

In an attempt to distinguish among *E. coli* serotypes exhibiting different phenotypes regarding production of the enzyme β -Gus, a multilayered paper sensor with two different indigo pigments duplex coloration was proposed by Kim et al.²² Without an additional cultivation step of *E. coli* but extra centrifugation and bacteria lysis steps, an LOD of $\sim 10^7$ CFU/mL was found by colorimetric analysis with a commercial scanner or bare eye visual detection. While smartphone detection by absorption of 4-nitrophenol on paper exhibited low values of the LOD of 2, 13 and 100 nM for β -glucosidase, β -galactosidase and β -Gus, respectively, the triggering bacteria *Enterococcus faecalis* was found only at a high concentration of 1×10^9 CFU/mL without pre-enrichment via culturing.²³ Thus, the simple

sensor system presented here is comparable regarding its LOD values to other sensoric systems based on enzyme chromogenic substrates that are under development, but does not require any extra lysis step or sensitive reagents.

3.3 Conclusion

In this study, we developed a paper-based biosensing substrate integrated with a smartphone-based readout system for the detection of β -Gus from *E. coli*. The enzymatic hydrolysis resulted in the formation of a distinct blue coloration, which served as a reliable indicator of enzyme activity. The limits of detection for visual assessment were 60 nM for neat enzyme solutions after 2 h of incubation, and $\sim 10^8$ CFU/mL for *E. coli* after 8 h. RGB analysis further elucidated the relationship between enzyme concentration, incubation time, and the intensity of colorimetric change, with the red channel acting as a significant indicator of product formation. The simplicity and efficacy of this smartphone-based paper biosensor in detecting β -Gus highlight its potential applications in environmental monitoring and clinical diagnostics, all while circumventing the need for complex preparation steps. Further optimization of this sensor could enhance its applicability across a broader range of bacterial types, thereby increasing its versatility and utility in various settings.

3.4 Materials and methods

3.4.1 Materials

Samples

Chitosan (medium molar mass, 190–310 kDa, 75–85% deacetylated), 5-bromo-4-chloro-3-indolyl- β -D-glucuronide (X-Gluc), *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC·HCl), *N*-hydroxy succinimide (NHS), β -glucuronidase (β -Gus) purified from *E. coli* (694.3 units/mg, E.C. 3.2.1.31; type IX-A), dimethyl sulfoxide (DMSO, 99%), pyridine (anhydrous, 99.8%), and phosphate buffer saline (PBS) were purchased from Sigma-Aldrich. *Escherichia coli* W (ATCC9637) derivative Mach1™ (T1 Phage-resistant, chemical competent, Invitrogen, USA), indigo (Carl Roth), acetic acid (glacial, J. T. Baker), Lysogeny broth (LB, Luria/Miller: 10g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7.0 $\pm$ 0.2, Carl Roth GmbH, Germany), qualitative filter paper (5-12 μ m and 12-15 μ m pore size, VWR, France) were purchased from the listed suppliers. Milli-Q water was drawn from a Millipore Direct Q8 system with a resistivity of 18.2 M Ω cm (Millipore advantage A10 system, Schwalbach, with Millimark Express 40 filter, Merck, Germany).

Sample preparation

1.5 wt% chitosan solution was prepared in 1 wt% acetic acid solution. 100 μL of chitosan solution was deposited on paper substrates ($0.8 \times 1.0 \text{ cm}^2$) and dried overnight. The two-time deposition of chitosan was done by pre-depositing 100 μL of chitosan solution on dried paper substrate followed by overnight drying in a flow hood. This chitosan deposition was repeated a second time.

Modification with 5-bromo-4-chloro-3-indole- β -D-glucuronide sodium salt (X-Gluc)

Chitosan-g-X-Gluc paper substrate was prepared using EDC/NHS chemistry according to the literature⁴. Briefly, 4.5 mM of X-Gluc was prepared in PBS (pH 7.4), followed by addition of EDC (6.7 mol/mol of X-Gluc) and NHS (6.7 mol/mol of X-Gluc). Then the solution was stirred for 1 h. Each chitosan sample was then immersed in 2 mL of this modification solution for 6 h under shaking (rate 60 Hz), followed by washing with Milli-Q water for 2 h under shaking, with subsequent exchange of Milli-Q water after each 30 min. Then the samples were dried overnight in a flow hood.

3.4.2 Methods

ATR-FTIR spectroscopy

A Bruker IFS 55 EQUINOX FTIR spectrometer was used for ATR-FTIR analysis. The chitosan coated paper was fixed on a holder and subsequently placed in the chamber of the instrument. The measurements were performed in absorbance mode in the spectral range 4000 to 400 cm^{-1} and a spectral resolution of 4 cm^{-1} . The background spectra were obtained by using air.

XPS analysis

The surface chemical composition of the films was analyzed by X-ray photoelectron spectroscopy (S-probe ESCA SSX-100s, Surface Science Instruments, USA) with an Al K α radiation of 200 W. Survey spectra were measured from 0 to 1200 eV with a resolution of 1 eV (spot size: 800 μm^2) and high resolution spectra with a resolution of 0.1 eV (spot size: 300 μm^2).

Enzymatic reaction with β -Glucuronidase

20 μM stock enzyme solution of β -Gus was prepared in sterilized PBS (pH 7.4) and the desired enzyme concentrations were prepared by further dilutions in PBS (pH 7.4) and LB (pH 7). 100 μL of the enzyme solutions tested were deposited on the paper sensors. Pictures were taken at different time intervals with Samsung Galaxy S8 camera with illumination in a black box, designed and produced by the workshop of the University of Siegen. The illumination was done with a LIVARNO LUX LED light strip (100-240 V, 50/60 Hz).

Detection of *E. coli*

For studying enzymatic cleavage via β -Gus produced by *E. coli* Mach1, overnight culture was prepared by taking 1 colony from an agar plate prepared in 5 mL LB and incubated at 37 °C for 18 h with shaking at 200 rpm. The OD_{600 nm} of overnight culture was set to 0.5 referring to a bacteria concentration of $(3 \pm 0.2) \times 10^8$ CFU/mL. Then the desired dilutions of bacteria suspension were prepared in LB. 100 μ L of each dilution of bacteria solution was deposited on the paper sensors for the experiment. All the experimental steps were performed at 20 °C and the pictures were taken at different incubation time intervals.

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4 Chitosan-based nanofiber sensing substrate

This Chapter details the development of a chitosan-based nanofiber sensing substrate for the improved detection of the enzyme β -Gus from pathogenic *E. coli*.ⁱ

4.1 Introduction and objectives

To overcome the limitations of conventional bacterial detection methods, significant attention has been focused on the development of cost-effective, rapid, accurate, and easy-to-use point-of-care tests for use in all healthcare settings. To address this need, numerous alternative methods have been developed, with a particular focus on nanomaterial-based approaches incorporating novel sensing materials such as nanoparticles,^{1,2} nanocapsules^{3,4} and reporter liposomes for the detection of bacterial infection in wound dressings^{3,5} as well as nanofiber-based sensors.^{6,7,8} Moreover, the use of colorimetric sensors utilizing enzyme-catalyzed reactions for autonomous reporting has emerged as an attractive and promising method for rapid bacteria detection.^{9,10,11,12} As a colorimetric sensing material, chitosan-based sensing hydrogels have extensively been studied for the rapid and selective detection and visual identification of bacteria.^{10,13,14}

To enhance the detection of *E. coli* using β -Gus as a bacterial marker, various nanoscale colorimetric sensing substrates have been developed. For instance, Das et al.¹⁵ synthesized enzyme-responsive nanogel fibers using nanopore extrusion observing a significant, but only marginally higher ($\sim 20\%$) enzymatic hydrolysis rate in the nanogel fibers compared to their hydrogel form. However, polymeric nanogels typically require complex fabrication processes,¹⁶ involving precise control of factors like temperature, pH, and crosslinking density. To overcome these issues, electrospun nanofibers have gained increasing attention due to their ease of functionalization, enhanced stability, and in particular scalability.¹⁷ Among various electrospun nanofibers, chitosan-based ones have been reported for their antibacterial and biomedical applications.^{18,19,20} Several colorimetric sensors made from electrospun nanofibers, including poly(ethylene oxide) and polyurethane,²¹ poly(vinyl alcohol-co-ethylene),²² and polyurethane^{23,24} have been studied for detecting bacteria, mainly non-pathogenic *E. coli*.

Chitosan-based electrospun nanofibers equipped with chromogenic substrates have recently been used as sensing substrates for bacteria detection, leveraging the unique properties of chitosan and the high surface area provided by electrospun nanofibers. Kinyua

ⁱ This work has been published in Polymer as: Kaur, K.; Schönherr, H. Enzyme-responsive chitosan-based electrospun nanofibers for enhanced detection of β -glucuronidase from pathogenic *E. coli*. *Polymer*, **2025**, 317, 127896. The figures were adapted from publication with permission.

et al.²⁵ reported the incorporation of a chromogenic sensing moiety in electrospun chitosan-based nanofibers for visual detection of β -Gus from non-pathogenic *E. coli* MACH-1. They observed 2.8 times faster enzymatic hydrolysis reaction rates with a chitosan nanofiber substrate compared to the corresponding hydrogel substrate. For an observation time of 60 min, they reported the limit of detection (LOD) of the enzyme β -Gus to be 13 nM using the nanofiber sensing substrate, compared to 25 nM with the hydrogel form.

This Chapter focuses on improving the sensitivity and performance of the electrospun nanofiber chromogenic sensing substrate, with the goal of enhancing the LOD of β -Gus detection. To achieve this, a potentially more sensitive fluorogenic substrate was incorporated into the electrospun chitosan-based nanofibers. Additionally, the efficacy of this sensing substrate will be assessed for detecting β -Gus produced by pathogenic *E. coli* NCTC 10418, an *E. coli* strain from the Enterobacteriaceae family that is listed among the WHO's priority pathogens for addressing AMR.²⁶

4.2 Results and discussion

Chitosan-based nanofibers were fabricated via electrospinning of solutions of chitosan and PEO and the hydrogel substrates were prepared using the drop-casting method. The morphology of the prepared substrates was characterized using SEM. The enzyme-sensitive nanofiber and hydrogel substrates were obtained by covalently attaching fluorogenic substrate MUG to chitosan using EDC/NHS chemistry. The modified substrates were then characterized using ATR-FTIR and ¹H-NMR spectroscopy. The enzymatic hydrolysis of the modified hydrogel and nanofibers was studied in the presence of enzyme β -Gus and bacterial suspensions. To investigate the effect of the nanofibrous morphology of CS-PEO on the sensing response after the hydrolysis reaction, the results were compared with those obtained from the CS-PEO hydrogel substrate.

4.2.1 Characterization of CS-PEO hydrogel and nanofibers

The morphologies of the synthesized CS-PEO hydrogels and nanofibers were characterized using SEM. Figure 1 shows the morphology of CS-PEO hydrogel and nanofiber substrates. The nanofibers were collected on a silicon wafer and the dried fiber webs could be readily peeled off from the wafer surface (Figure 1c). A thin layer of nanofiber sheet was obtained after peeling from the wafer surface. Assuming the nanofibers to be cylindrical, the surface area can be estimated when the mass and fiber diameter are known. For 3.00 mg sample, the nanofibers have an estimated surface area of 323 cm². For a hydrogel of the same mass, the surface area was determined to be 0.500 cm² by measuring the dimensions of the sample.

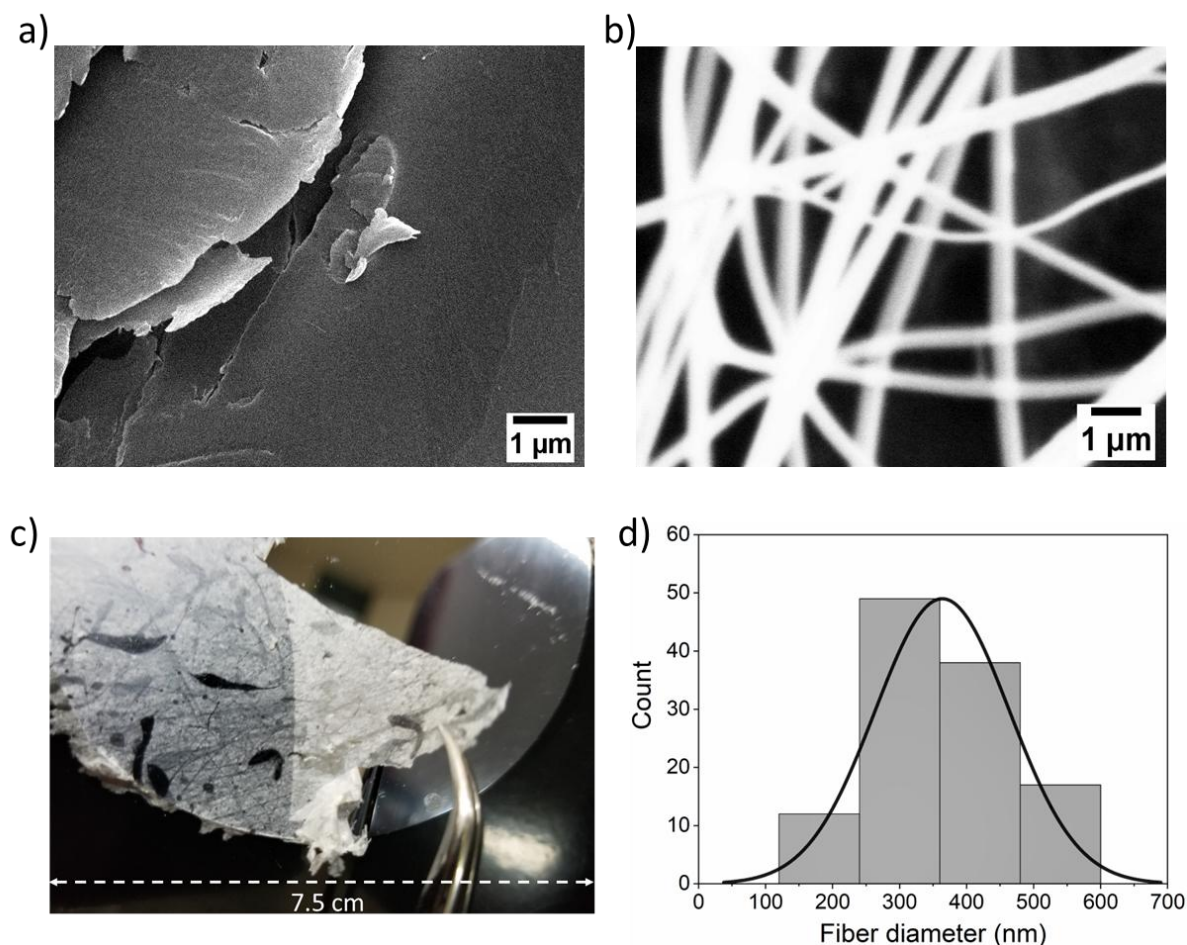


Figure 1: SEM images of CS-PEO a) hydrogel and b) nanofiber substrate. c) Photograph of dried electrospun nanofiber mat that is being removed from the silicon wafer. d) Histogram of fiber diameter of the nanofiber sample depicted in panel b), electrospun at a flow rate of 15 $\mu\text{L}/\text{min}$, an applied voltage of 20 kV, and a tip-to-collector distance of 15 cm.

It is well established that the electrospinning parameters, including polymer solution properties (such as polymer molar mass, concentration, viscosity, and conductivity) and process parameters (including applied voltage, flow rate, and tip-to-collector distance) as well as environmental factors (such as temperature and humidity) significantly influence the diameter and morphology of the resultant nanofibers.²⁷ The electrospinning process parameters were systematically varied in our study to investigate their effects on the fiber diameters and the morphology, as shown in Figure 2. A typical electrospinning set-up was used as shown in Figure 2a. To determine the fiber diameter of electrospun CS-PEO nanofibers fabricated at different electrospinning conditions, an analysis was conducted on 80-100 fibers using ImageJ software. For each varied condition, the fiber diameter was determined from three separate samples, with measurements taken at different positions to check the uniformity of nanofiber formation.

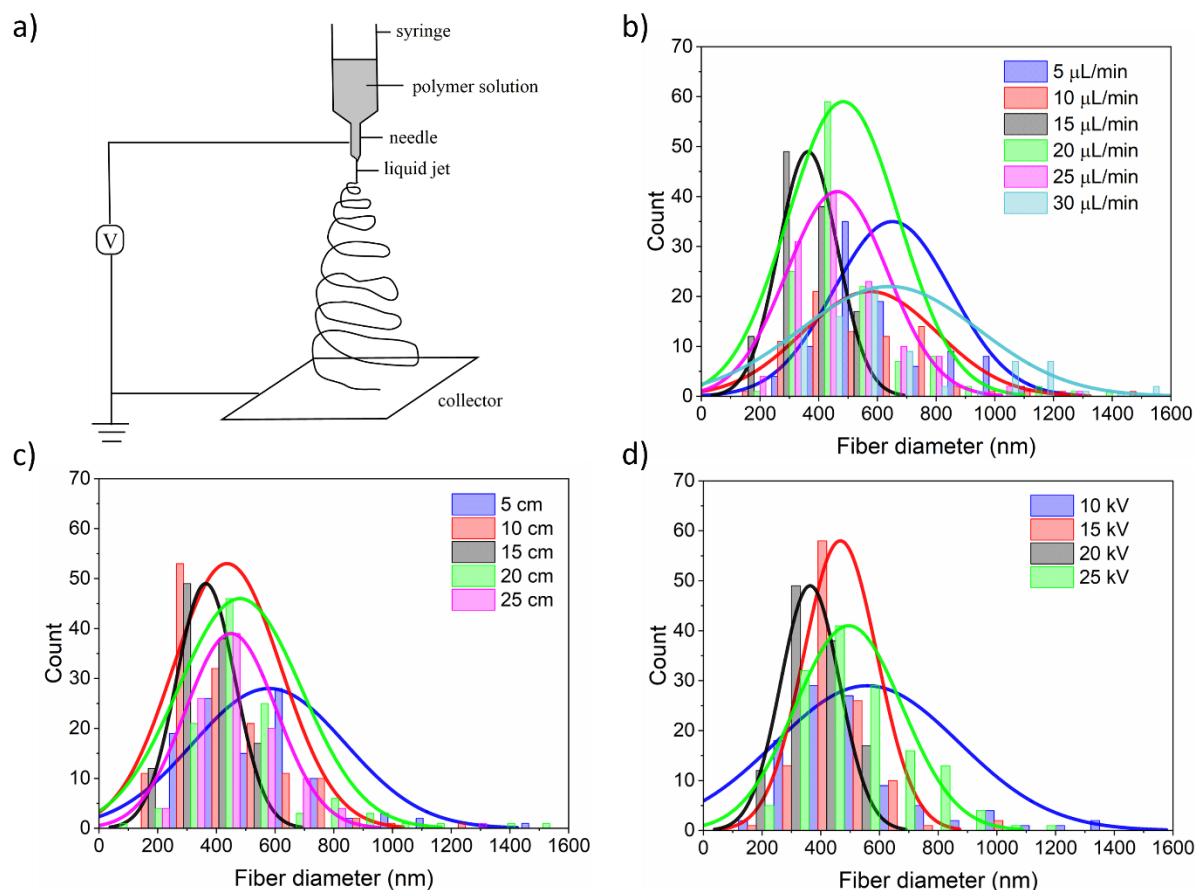


Figure 2: a) Scheme of electrospinning setup. Histogram of fiber diameters of electrospun nanofibers obtained from CS-PEO solutions with varied b) flow rate (@ 20 kV, 15 cm), c) tip-to-collector distance (@ 20 kV, 15 $\mu\text{L}/\text{min}$), and d) applied voltage (@ 15 cm, 15 $\mu\text{L}/\text{min}$).

Very few fibers containing beads were observed at a flow rate of 5 $\mu\text{L}/\text{min}$ (Figure 2b). However, when the flow rate was increased to 10 $\mu\text{L}/\text{min}$, the bead formation was less prominent. At a flow rate of 15 $\mu\text{L}/\text{min}$, a highly homogenous fiber mat was observed on the collector. However, increasing the flow rate further resulted in the formation of thicker fibers containing beads. The sample collected at a flow rate of 15 $\mu\text{L}/\text{min}$ exhibited a narrow distribution of fiber diameters. By contrast, other flow rate values showed significant deviations in fiber diameters, indicating a wider distribution range of fiber diameters.

For varied tip-to-collector distances (Figure 2c), it was observed that the fiber diameter decreases with increasing distance from the collector. At a shorter distance of 5 cm, it was challenging to collect fibers due to the formation of sparks during electrospinning. Homogenous fibers were observed at a tip-to-collector distance of 15 cm. When the distance was further increased, fewer fibers were collected and the distribution range of diameters became wider.

Additionally, the effect of varied applied voltage on the formation of nanofibers was analyzed (Figure 2d). At lower voltages, thicker fibers were produced, whereas higher voltages

resulted in finer fibers with varied diameters. At applied voltages below 15 kV, very few fibers were produced and the polymer jet tended to fly to the sides of the collector, resulting in fibers with a large distribution range of diameters. A well-formed Taylor cone was observed at an applied voltage of 20 kV and homogenous fibers with a narrow diameter distribution were collected. Increasing the applied voltage beyond this point led to the production of sparks, causing the non-continuous formation of fibers. Thus, by carefully controlling the electrospinning parameters, the properties of electrospun nanofibers can be tailored to meet specific application requirements.

The nanofibers electrospun at a flow rate of 15 $\mu\text{L}/\text{min}$, an applied voltage of 20 kV, and a tip-to-collector distance of 15 cm were used for subsequent studies. Following the preparation of CS-PEO nanofiber and hydrogel substrates, the fluorogenic enzyme sensing moiety MUG was incorporated into both substrates using EDC/NHS chemistry (Figure 3). During this reaction, the carboxylic acid group of the fluorogenic substrate is covalently coupled to the primary amine group of the glucosamine group in chitosan, forming a stable amide bond.

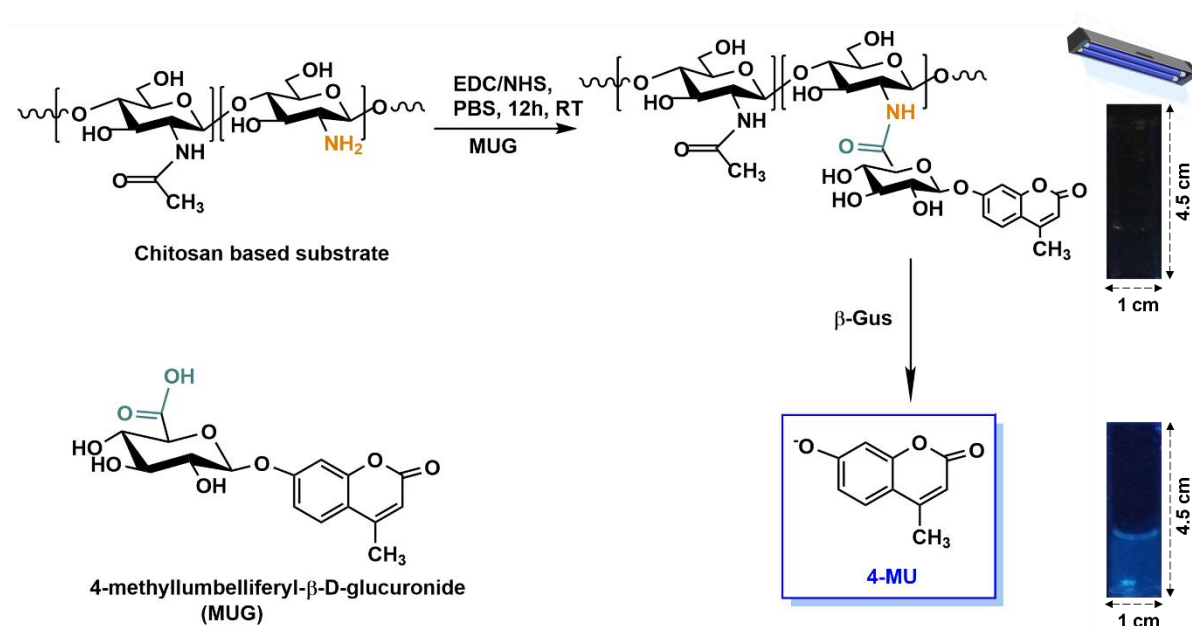


Figure 3: Schematic of the chemical modification of chitosan-based nanofiber and hydrogel substrates with the fluorogenic dye substrate MUG via EDC/NHS chemistry. Following enzymatic hydrolysis catalyzed by β -Gus the reporter dye 4-methylumbelliferone (4-MU) is liberated. The blue emission of the released 4-MU can be observed by illumination under a hand-held UV lamp ($\lambda_{\text{ex}} = 365 \text{ nm}$).

The resulting MUG functionalized chitosan-based nanofibers and hydrogels were characterized by using ATR-FTIR spectroscopy. Figure 4 shows the ATR-FTIR spectra of chitosan and PEO, CS-PEO hydrogels and nanofibers, and modified CS-PEO hydrogels and nanofibers.

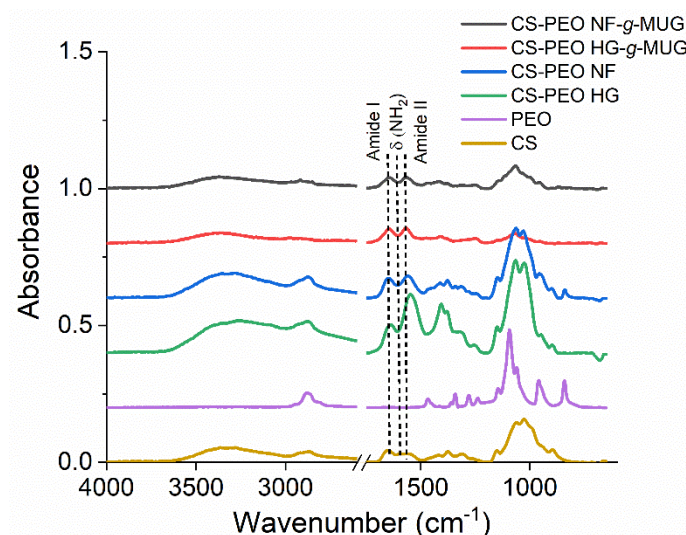


Figure 4: ATR-FTIR spectra of the MUG-modified and non-modified hydrogel and nanofibers.

All important vibration bands of chitosan, i.e. the primary amine (NH_2), amide I ($\text{C}=\text{O}$) and amide II ($\text{N}-\text{H}$) bands were observed around 1591 cm^{-1} , 1640 cm^{-1} , 1558 cm^{-1} , respectively, for unmodified CS-PEO nanofibers and at 1593 cm^{-1} , 1643 cm^{-1} , 1548 cm^{-1} , respectively, for unmodified CS-PEO hydrogel. In the modified nanofibers and hydrogel, the $-\text{NH}_2$ band intensity was drastically reduced and the intensities of the amide I and amide II bands decreased, indicating the amino group's participation in the chemical reaction. The assignment of the observed bands is summarized in Table 1. The spectrum of neat PEG shows the vibrations associated to semicrystalline PEG.²⁸ The absence of these bands in the spectra of the nanofiber samples suggests low content, if any, comprising partially crystalline domains of PEG.

Table 1: Band assignment of the important bands observed in the ATR-FTIR spectra of modified and non-modified hydrogel and nanofibers.

Assignment	Band position (cm^{-1}) Reference Chitosan ⁹	Band position (cm^{-1}) Non-modified		Band position (cm^{-1}) Modified	
		NF	HG	NF	HG
N-H (amide II)	1550-1565	1558	1548	1558	1544
NH_2	1590-1610	1591	1593	-	-
C=O (amide I)	1620-1655	1640	1643	1649	1644

The successful modification with the dye substrate MUG was also confirmed through ^1H -NMR spectroscopy analysis. In the spectrum of CS-PEO (Figure 5), the chemical shifts of the

protons from *N*-acetyl appeared at 1.85 ppm. The multiplet observed between 3.46 and 3.80 ppm is attributed to the chemical shifts of protons on the CS backbone. The singlets at 2.96 ppm and 4.37 ppm correspond to protons from the -CH-NH₂ and -CH-O groups in the glucosamine unit, respectively.²⁹ A small peak at 8.08 ppm was observed, corresponding to the proton attached to NH-C=O of the acetylated end.

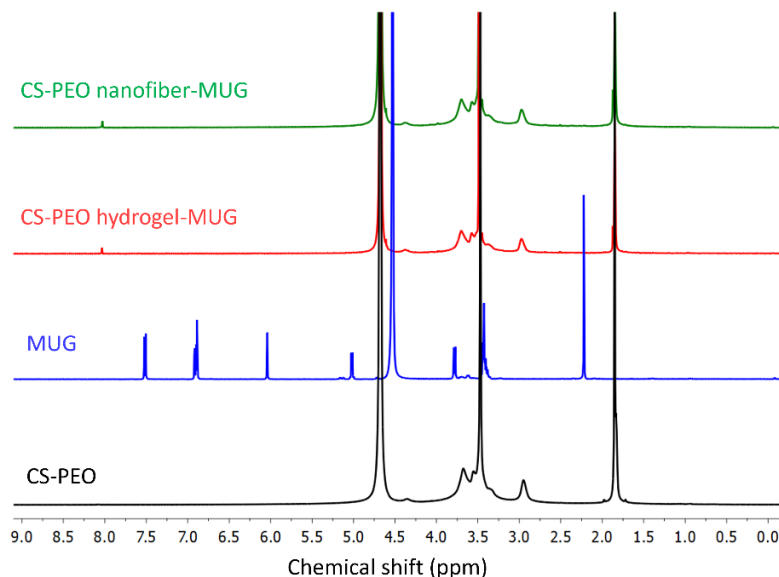


Figure 5: ¹H-NMR spectra of CS-PEO, fluorogenic substrate MUG, modified CS-PEO hydrogel, and nanofiber in 1% CD₃COOD solvent in D₂O. The spectra were referenced to 0 ppm corresponding to tetramethylsilane. The singlet at 4.53 ppm in MUG and at 4.68 ppm in CS-PEO, CS-PEO hydrogel-MUG and CS-PEO nanofiber-MUG correspond to protons from the non-deuterated solvent molecules.

Following the reaction with the dye substrate MUG, all the signal peaks corresponding to chitosan were observed in the ¹H-NMR spectra of the modified CS-PEO hydrogels and nanofibers (Figure 6).

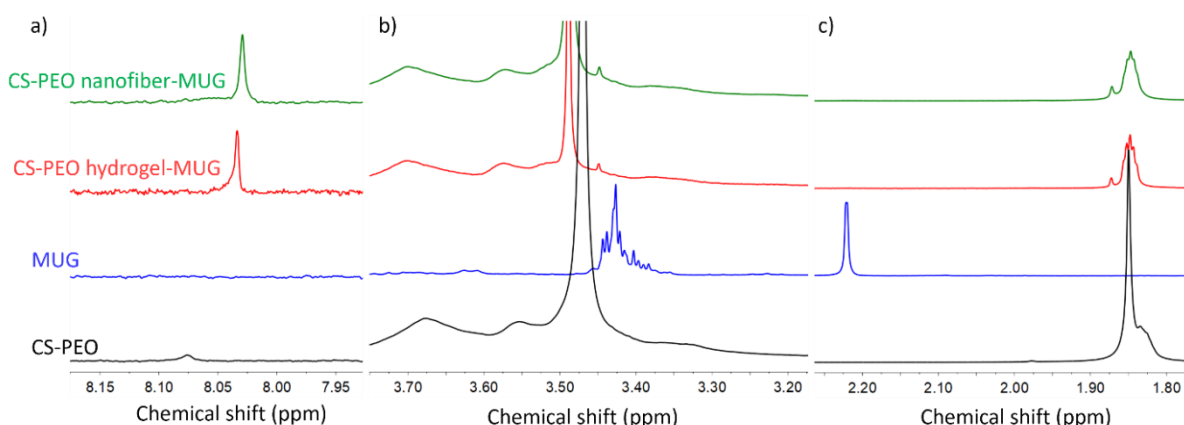


Figure 6: ¹H-NMR spectrum of CS-PEO, the fluorogenic substrate MUG, modified CS-PEO hydrogel, and nanofiber in 1% CD₃COOD in D₂O zoomed in at the desired peak positions confirming successful coupling of MUG to the substrates.

The shift of the peak at 8.08 ppm to 8.03 ppm in the modified CS-PEO hydrogel and the nanofibers (Figure 6a) indicates the formation of new amide bonds in the reaction. Moreover, a new peak at 3.45 ppm, corresponding to protons from the glucuronide unit of MUG (Figure 6b), and a sharp peak at 1.87 ppm corresponding to $-CH_3$ proton from MUG appeared (Figure 6c). In the spectrum of MUG, the peak at 2.22 ppm, corresponding to the protons of $-CH_3$ group of the 4-MU moiety, was identified as the signature peak.

The reference deconvolution method was used to plot the calibration curve using the area under this peak vs known MUG concentration. Figure 7 shows the resulting calibration curve. After the modification reaction, this peak of MUG shifted and appeared at 1.87 ppm in the modified samples. Thus, this peak was deconvoluted to determine the unknown MUG concentration in modified samples. The estimated MUG concentration in modified CS-PEO nanofibers and hydrogels was found to be 5.7 mg/mL and 3.6 mg/mL, respectively.

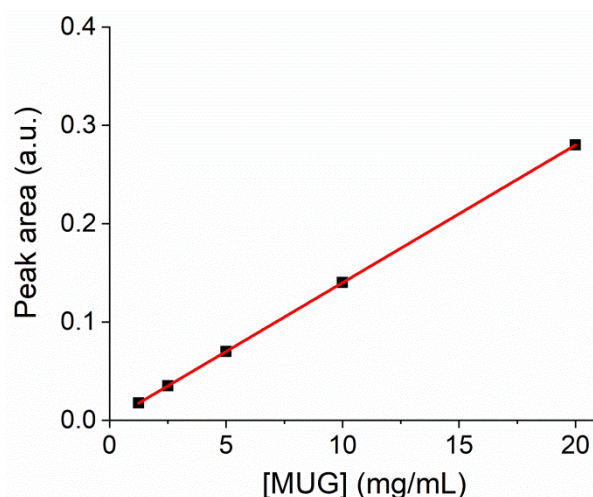


Figure 7: The calibration curve of the dye substrate MUG prepared from ^1H spectrum of MUG prepared in 1% CD_3COOD solvent in D_2O . The data was fitted with linearly.

4.2.2 Enzymatic hydrolysis of MUG-modified CS-PEO hydrogel and nanofibers

The enzymatic reactions of the modified nanofibers and hydrogels were performed in a neat buffered β -Gus solution at pH 7.40 under ambient conditions. The MUG-modified CS-PEO nanofibers and hydrogel contain the coumarin derivative 4-MU linked via β -glycosidic linkages. The enzyme β -Gus cleaves 4-MU from the glucuronide unit via hydrolytic cleavage of MUG residues (Figure 3). The enzymatic reaction of the modified nanofibers and hydrogels was monitored using sequential measurements with a fluorescence spectrometer. The increase in fluorescence emission at 450 nm over time provided confirmation of the liberation of 4-MU from the substrates. Figure 8 shows the kinetic measurements of the modified nanofiber and hydrogel substrate in the presence of 100 nM β -Gus recorded over time. These measurements

showed that the fluorescence emission of the released 4-MU dye increased progressively during the enzymatic reaction.

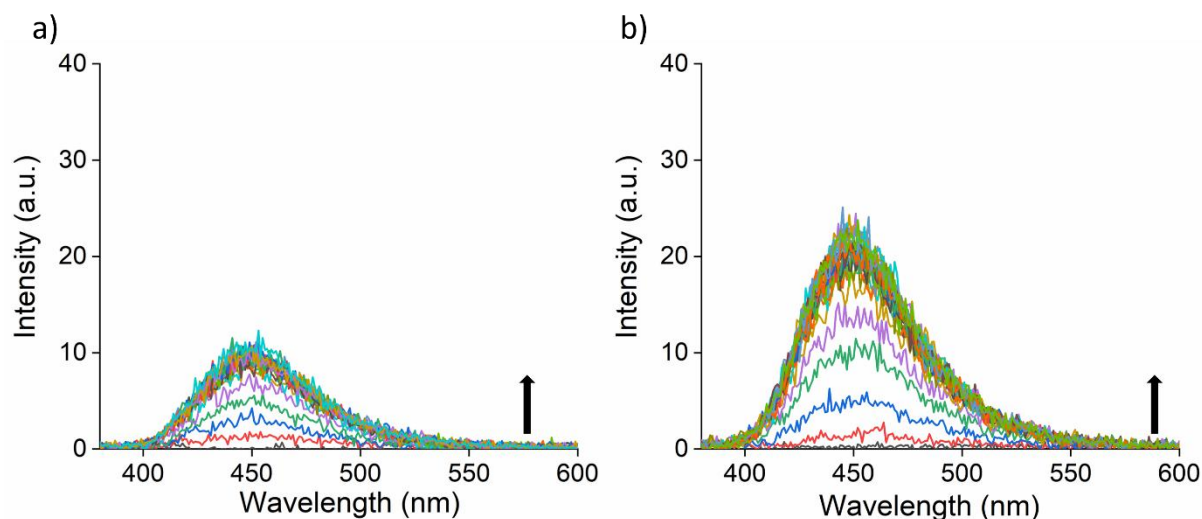


Figure 8: Fluorescence spectra of the enzymatic reaction of 3 mg a) MUG-modified CS-PEO hydrogel and b) MUG-modified CS-PEO nanofibers in 100 nM β -Gus (λ_{ex} = 365 nm with a measurement repeat of 3 min) for 120 min at 25°C.

Under the same reaction conditions, the modified nanofibers exhibited a higher signal intensity of 4-MU compared to the hydrogel substrate. As both the dried modified hydrogel and nanofibers are insoluble in the enzyme solution, the enzymatic reaction initially occurs at the substrate surface. Upon swelling in the enzyme solution, the nanofiber network tends to loosen up facilitating the diffusion of the enzyme into the three-dimensional network of fibers.¹⁵ Figure 9 shows the fluorescence emission of 4-MU as a function of reaction time for the initial reaction of modified a) hydrogel and b) nanofibers in experiments, in which the enzyme concentration was varied from 5 nM to 200 nM.

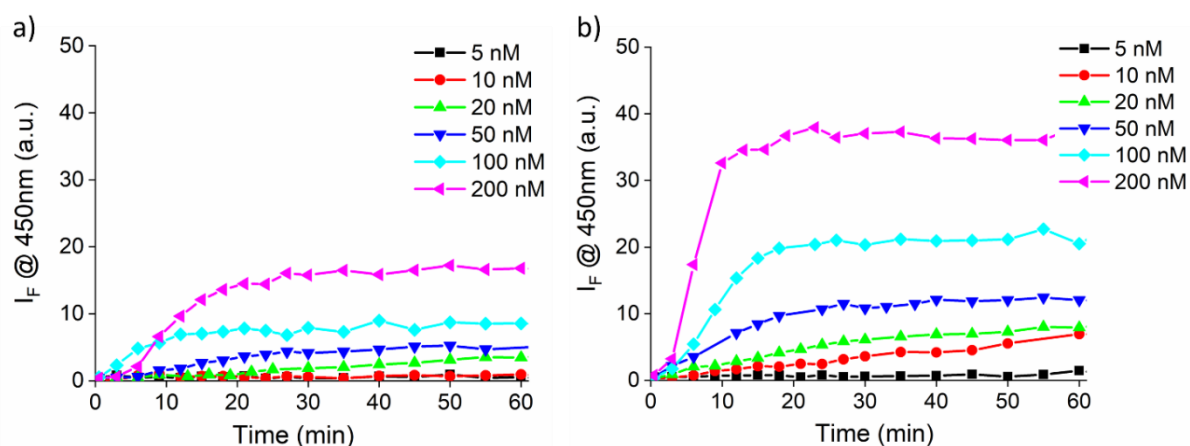


Figure 9: Fluorescence kinetics plots of the MUG-modified CS-PEO a) hydrogel and b) nanofibers in the presence of different enzyme concentrations (λ_{ex} = 365 nm with a measurement repeat of 3 min).

In both the nanofiber and hydrogel substrates, an increase in enzyme concentration enhances the release of 4-MU, until it reaches a plateau after some time. The reaction kinetics was recorded for a total of 240 min and no further release of dye was observed after reaching the plateau (Figure 10).

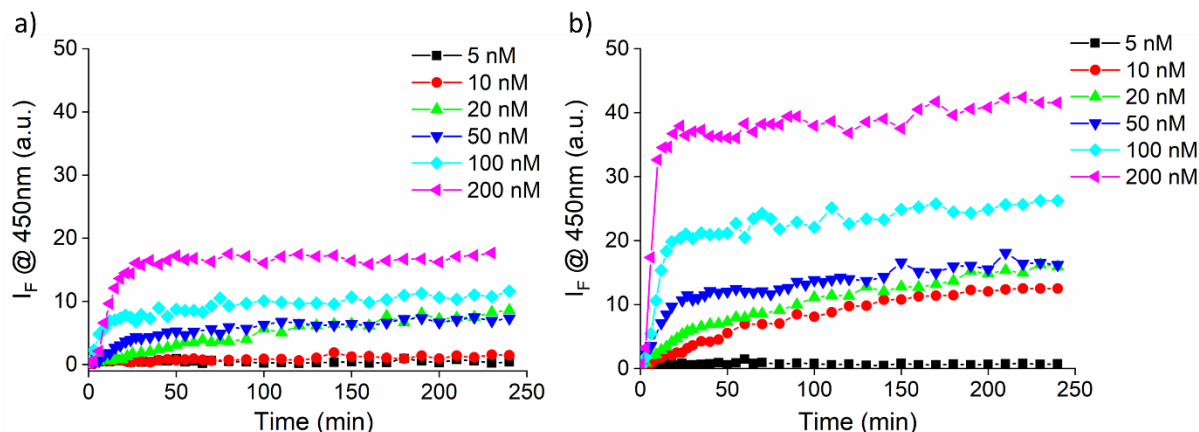


Figure 10: Fluorescence kinetics plots of the MUG-modified CS-PEO a) hydrogel and b) nanofibers in the presence of β -Gus at different concentrations in buffer, studied for 240 min at $\lambda_{ex} = 365$ nm with a measurement repeat of 3 min.

For lower enzyme concentrations more time is required to reach the plateau, indicating fewer available enzyme sites to bind with the substrate and release the dye. Conversely, higher enzyme concentrations facilitate a faster conversion of the substrate into the product within the same timeframe. This remains applicable as long as the enzyme concentration serves as the limiting factor for the ongoing reaction. Initially there is sufficient substrate available to bind with the enzyme's active sites, however as the reaction proceeds and the substrate is depleted, further increasing the enzyme concentration will not affect the amount of released dye.

Additionally, a standard curve of 4-MU in PBS (pH 7.40) was recorded to determine the concentrations of released 4-MU following the enzymatic reactions (Figure 11).

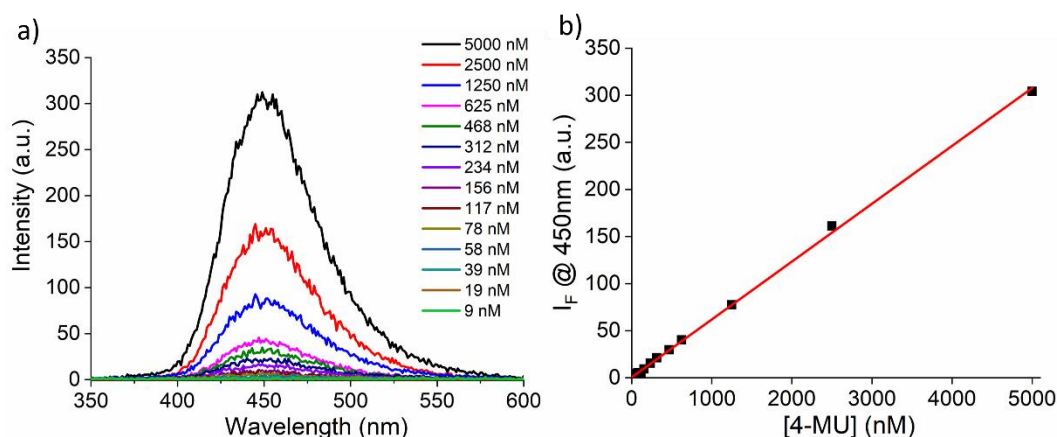


Figure 11: a) Fluorescence spectra of 4-MU measured at $\lambda_{ex} = 365$ nm for different concentrations in PBS (pH=7.4), b) calibration curve of 4-MU in PBS (pH= 7.4). The data were fitted with a linear least squares fit.

The LOD and limit of quantification (LOQ) of 4-MU were calculated to be 2.85 nM and 8.31 nM, respectively. The quantity of released dye 4-MU after 240 min of the enzymatic reactions in the presence of various enzyme concentrations was also calculated and is given in Table 2. Specifically in the presence of 200 nM β -Gus, 280 nM of 4-MU was released in the presence of modified hydrogel, while 669 nM of 4-MU was released in the presence of modified nanofiber after 240 min of the enzymatic reaction.

Table 2: The concentrations of released 4-MU after 240 min of the enzymatic hydrolysis of the modified hydrogel and nanofibers studied with different enzyme concentrations.

β -Gus concentration (nM)	Released 4-MU (nM)	
	Modified hydrogel	Modified nanofiber
5	0.215	4.11
10	16.2	195
20	132	251
50	111	256
100	181	419
200	279	669

Figure 12 shows the effect of enzyme concentration on the initial apparent reaction rates observed during the enzyme kinetics of modified hydrogel and nanofibers. The initial apparent reaction rate was defined as the slope of the enzymatic kinetics plot in the first 15 min and was obtained from a linear least-squares fit.

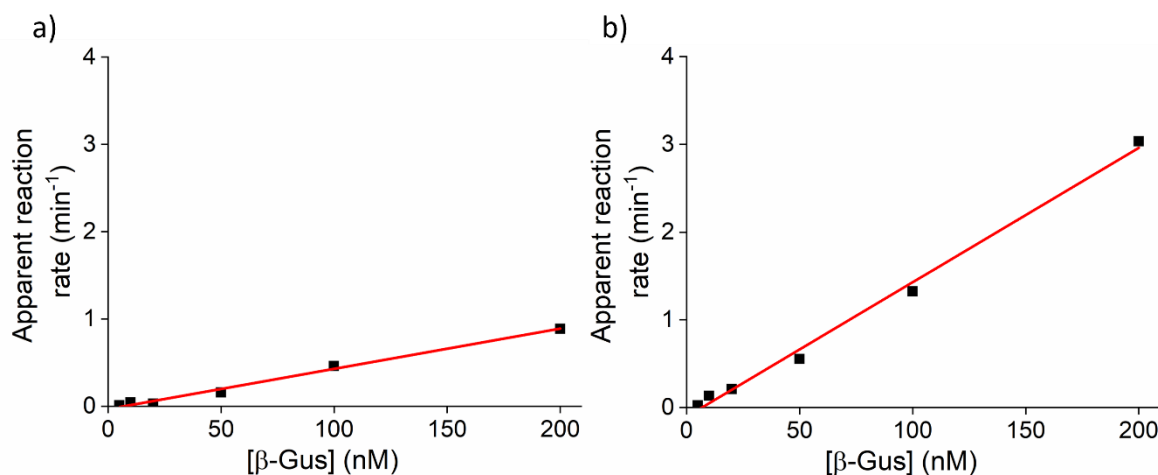


Figure 12: The initial apparent reaction rates as a function of enzyme concentrations after the enzymatic hydrolysis of modified a) hydrogel and b) nanofibers. Linear least square fitting of data yielded intercept values of $-0.032 \pm 0.019 \text{ min}^{-1}$ for hydrogel in panel a) and $-0.105 \pm 0.054 \text{ min}^{-1}$ for nanofibers in panel b).

For both hydrogel and nanofibers, the initial apparent reaction rate for up to 15 min was observed to increase linearly with the enzyme concentration, which is in agreement with the Michaelis-Menten kinetics.^{30,31,32} It was also observed that the rate of enzymatic hydrolysis was higher for the nanofibers compared to the hydrogel. Table 3 compiles the initial apparent reaction rates observed at different enzyme concentrations for modified hydrogel and

nanofibers. For 200 nM β -Gus, the initial apparent reaction rate was observed to be $0.88 \pm 0.07 \text{ min}^{-1}$ for modified hydrogel and $3.03 \pm 0.38 \text{ min}^{-1}$ for modified nanofibers.

Table 3: The initial apparent reaction rates observed at different enzyme concentrations after the enzymatic hydrolysis of the modified hydrogel and nanofibers.

β -Gus concentration (nM)	Initial apparent reaction rate (min^{-1})	
	Modified hydrogel	Modified nanofiber
5	0.01 ± 0.01	0.02 ± 0.04
10	0.04 ± 0.02	0.13 ± 0.01
20	0.03 ± 0.02	0.21 ± 0.02
50	0.16 ± 0.02	0.55 ± 0.02
100	0.46 ± 0.07	1.32 ± 0.08
200	0.88 ± 0.07	3.03 ± 0.38

The MUG-modified CS-PEO nanofibers showed ~ 3.4 times faster enzymatic hydrolysis indicating to be a more sensitive sensing substrate toward β -Gus detection than the hydrogel formation of CS-PEO. The enhanced initial reaction rates in the case of sensing nanofibers can be associated with the higher surface-to-volume ratio.²⁵ The higher the surface area to volume ratio, the more substrate molecules are available to the enzyme resulting in higher initial reaction rates and hence faster sensor response. However, the enhancement factor is 3 orders of magnitude smaller than estimated assuming a proportional increase in rate with increasing surface area. Thus, the swelling of neat MUG-functionalized chitosan hydrogel, which is known to be with a ratio of 6 - 7 substantial, must be the most important factor to afford access of the enzyme to the substrate conjugated to chitosan.²⁵ As hypothesized, changing the morphology to increase the surface-to-volume ratio may result in a significant enhancement. Hence, increasing the specific surface area of the sensor is an efficient strategy to enhance the sensor performance.

Qualitatively similar findings of enhanced reaction rates and sensitivity in nanofibers compared to hydrogels to detect bacterial enzyme β -Gus have been reported in the literature. Kinyua et al.²⁵ reported 2.8 times faster enzymatic hydrolysis reaction rates with chitosan nanofibers compared to the corresponding hydrogels. Here, we observed 3.4 times increase, suggesting that morphology control by electrospinning may also add to the enhancement.

Moreover, these enzymatic hydrolysis results can be compared to those of chitosan-based hydrogels reported in the literature. Jia et al. synthesized β -Gus enzyme-responsive chitosan hydrogels and reported rate of enzymatic reactions of $2.5 \times 10^{-3} \text{ min}^{-1}$ (in first 30 min) and $1.1 \times 10^{-4} \text{ min}^{-1}$ (in first 40 min) in the presence of 1 mM β -Gus when chitosan conjugated with 5-bromo-4-chloro-3-indolyl- β -D-glucuronide (X-Gluc) and 4-nitrophenyl- β -D-glucuronide (PNPG) respectively.¹¹ In another report, they observed slightly higher initial apparent rates ($t = 30 \text{ min}$) of $3.29 \times 10^{-3} \text{ min}^{-1}$ and $2.50 \times 10^{-3} \text{ min}^{-1}$ in presence of 2 μM β -Gus for shape-encoded chitosan hydrogels conjugated to different concentrations of chromogenic substrate

X-Gluc¹³ and an apparent rate constant ($t \leq 30$ min) of $0.034 \pm 0.002 \text{ min}^{-1}\text{mM}^{-1}$ in the presence of $0.2 \text{ } \mu\text{M}$ β -Gus for color-encoded chitosan hydrogel conjugated to PNPG.³³ In all these instances, slower reaction rates were observed, and significantly higher enzyme concentrations were used to enhance the reaction rates.

Further increasing the surface area available for enzymatic attack can in principle be realized in nanoparticles. However, polymeric nanogels involve typically complex fabrication processes³⁴ requiring precise control over conditions like temperature, pH, and crosslinking density. To overcome these issues, electrospun nanofibers are increasingly favored due to their ease of functionalization, enhanced stability, and in particular scalability.¹⁷

LOD of enzyme β -Gus

The LOD of an enzyme is defined as the lowest concentration of the enzyme that can be detected depending on the release of the minimum detectable amount of the released dye. This can be estimated using the fluorescence intensity values corresponding to the LOD for the detection of the dye (4-MU) divided by the value of the rate constant (slope of the plot apparent initial reaction rate vs enzyme concentration) multiplied by the observation time. The LOD 4.4 nM of 4-MU in the presence of unreacted fluorogenic substrate MUG has already been reported in previous reports³¹ and was used to determine the LOD of β -Gus. Figure 13 shows the calculated LODs of β -Gus at different reaction times when studied with hydrogel substrate (Figure 13a) and in the presence of nanofiber substrate (Figure 13b).

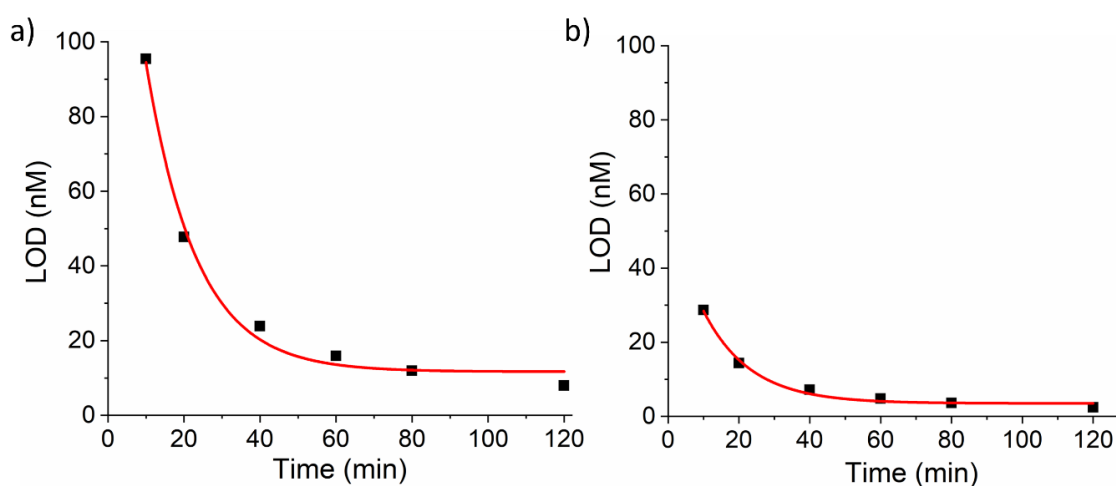


Figure 13: The limit of detection of β -Gus observed at different reaction times in the presence of modified a) hydrogel and b) nanofibers. The data were fitted with a monoexponential decay.

The LOD of β -Gus decreases approximately exponentially with observation time. Hence depending on the observation time LOD of the enzyme differs. After 60 min of observation time, the LOD of β -Gus was observed to be 16 nM for the CS-PEO hydrogel and 4.7 nM for the CS-PEO nanofiber sensing substrates. This confirms again that the nanofiber sensing

substrate was observed to be more sensitive toward β -Gus detection compared to the hydrogel substrate.

Kinyua et al.²⁵ reported that after 60 min observation time using a chromogenic substrate, the LOD of β -Gus was 25 nM with CS-PEO hydrogel and 13 nM with CS-PEO nanofiber. Hence, it can be concluded that the LOD of β -Gus has been improved by 2.8 times for the nanofiber substrate and 1.6 times for the hydrogel when modified with the fluorogenic substrate MUG.

The LODs of β -Gus have also been reported by Jia et al., who prepared autonomously reporting chitosan hydrogels coupled with chromogenic substrates. For an observation time of 60 min, they reported a β -Gus LOD of ≤ 5 nM¹³ and a LOD of 4.5 nM³³ when analyzed with a spectrometer and microplate reader, respectively. In both experimental conditions, the chitosan solution was deposited onto supporting substrates to form a thin hydrogel film. This film was then modified with a chromogenic dye and analyzed under conditions involving enzyme concentrations higher than those used in the current study. Hence similar LOD's can be achieved with lower enzyme concentrations using CS-PEO nanofiber sensing substrates.

***In vitro* detection of β -Gus**

Furthermore, the MUG-modified CS-PEO nanofiber and hydrogel substrates were used for *in vitro* detection of β -Gus in the presence of *E. coli* suspensions. The enzyme β -Gus is known to be secreted by more than 98% of all known *Escherichia coli* (*E. coli*) strains.³¹ In this study two bacterial strains were tested: the pathogenic *E. coli* NCTC 10418 and the non-pathogenic *E. coli* MACH1-T1. Figure 14 shows the *in vitro* experimental results of the MUG-modified CS-PEO nanofibers and hydrogel in the presence of *E. coli* MACH1 (Figure 14a) and NCTC (Figure 14b).

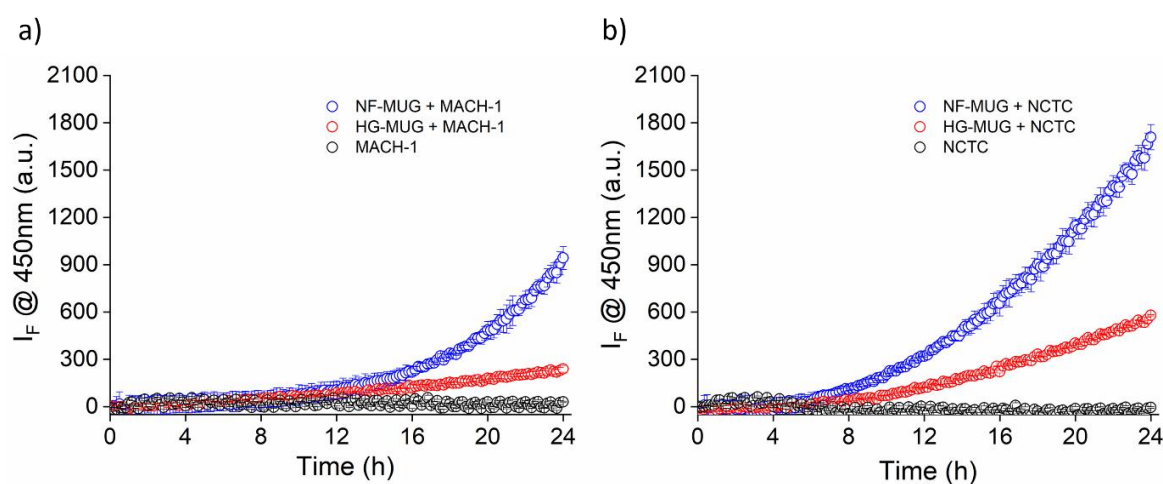


Figure 14: Fluorescence kinetics plot of the MUG-modified CS-PEO hydrogel and nanofibers in the presence of a) *E. coli* MACH-1 and b) *E. coli* NCTC studied for 24 h at $\lambda_{ex} = 365$ nm with measurement repeat 10 min.

The fluorescence intensity of the released dye 4-MU at 450 nm remained almost constant over time for both the bacterial suspensions in LB medium. However, an increase in fluorescence signal was observed for the bacterial suspensions containing MUG-modified hydrogel and nanofibers. This increase in intensity indicates that the enzyme β -Gus produced by *E. coli* suspensions is catalyzing the hydrolysis of β -glycosidic linkages in the modified nanofibers and hydrogel, resulting in the release of 4-MU into the medium. Among both bacterial suspensions, the modified nanofibers exhibited a higher increase in signal intensities compared to the modified hydrogel. This confirms again that the CS-PEO nanofiber sensing substrate is more sensitive to β -Gus detection than its hydrogel form. When comparing the two bacterial suspensions, higher signal intensities of released dye were observed for the strain NCTC than the MACH-1. The control samples including non-modified hydrogel and nanofibers in the presence of LB and bacterial suspensions, as well as the modified samples measured in the presence of LB alone, showed no increase in fluorescence intensity at 450 nm (Figure 15). Therefore, the prepared CS-PEO nanofiber sensing substrate successfully detects both the pathogenic and non-pathogenic *E. coli* strains producing β -Gus. The nanofiber sensing substrate was found to be ~ 4 times more sensitive than the hydrogel sensing substrate in the presence of MACH-1 and ~ 3 times more sensitive in the presence of NCTC.

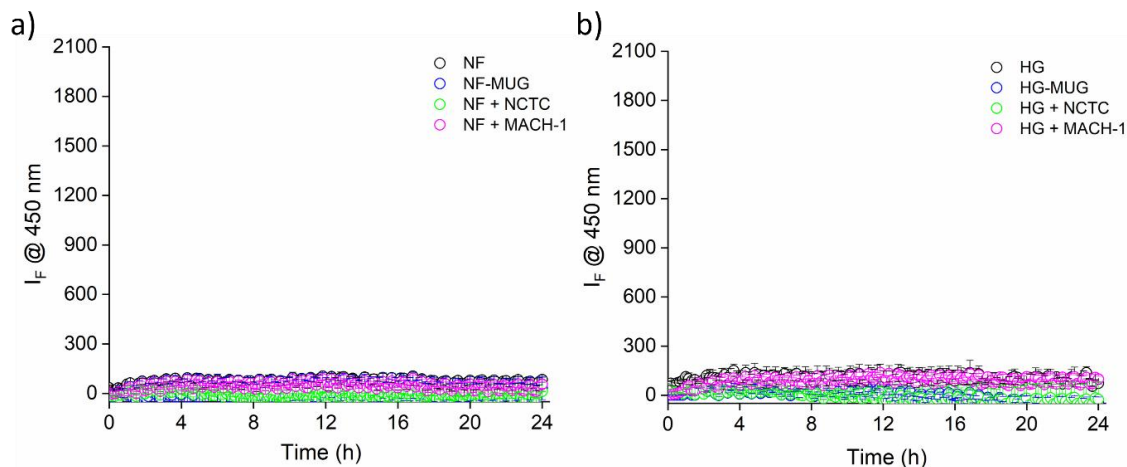


Figure 15: Fluorescence kinetics plot of the non-modified and modified a) nanofibers and b) hydrogels in the bacteria suspensions and LB medium respectively (24 h kinetics at $\lambda_{ex} = 365$ nm with a measurement repeat 10 min).

The CS-PEO nanofiber-based enzyme sensing substrate reported here eliminates the need for an additional supporting substrate, producing a free-standing enzyme sensing substrate. This substrate achieves similar LODs for β -Gus using lower enzyme concentrations than those reported in the literature. The LOD value obtained using this nanofiber-based sensing substrate can be further improved by optimizing the fiber diameters thereby enhancing its

sensing capabilities. Free-standing nanofibers do not require a supporting substrate which simplifies their handling and integration into devices, resulting in reduced preparation steps and potential sources of contamination.³⁵ Moreover, they provide an increased surface area, improved mechanical properties and flexibility,³⁶ reduced swelling and shrinking issues,³⁷ scalability, and ease of fabrication for producing large quantities for industrial applications³⁸ compared to hydrogels on substrates, making them a better choice for various applications including sensing and biomedical devices.

4.3 Conclusion

Autonomously reporting chitosan-based nanofibers with an average fiber diameter of 363 ± 100 nm for β -Gus detection were successfully fabricated by electrospinning and modification with the fluorogenic substrate MUG, demonstrating significantly enhanced sensitivity and practical advantages in fabrication and scalability. At a fixed β -Gus concentration, the nanofibers exhibited a 3.4-fold increase in the initial enzymatic hydrolysis rate over the hydrogels and exhibited a significantly lower LOD for β -Gus, reaching 4.7 nM after 60 min compared to 16 nM for hydrogels. These data confirm the superior sensitivity of nanofibers for enzyme detection, which was demonstrated in *in vitro* experiments with both pathogenic and non-pathogenic *E. coli* strains, highlighting their broad applicability in real biological environments. These findings underscore the nanofiber substrates' potential for advanced biosensing and diagnostic applications, where rapid and sensitive enzyme detection is critical.

4.4 Materials and methods

4.4.1 Materials

Samples

Chitosan (CS, medium molar mass, 190–310 kDa, 75–85% deacetylated), polyethylene oxide, PEO ($M_w > 5000$ kg/mol), *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC.HCL, $\geq 99\%$), β -glucuronidase from *E. coli* (β -Gus, 694.3 units/mg, E.C. 3.2.1.31; type IX-A, lyophilized powder, 25,000 units/g protein), trichloro octadecyl silane ($\geq 90\%$), phosphate saline buffer solution (PBS, pH 7.4), 4-methylumbelliferyl- β -D-glucuronide (MUG), *N*-hydroxy succinimide (NHS) and deuterated acetic acid (CD_3COOD) were purchased from Sigma-Aldrich, Germany. Acetic acid (glacial, J. T. Baker, Germany), sodium hydroxide (NaOH, $>98.8\%$, ChemSolute), disposable acrylic cuvettes (10 mm $\times$ 10 mm $\times$ 45 mm, Sarstedt, Germany), syringe (10 mL, Henke-Ject, Germany), needle (21 G, Braun, Germany), deuterated water (D_2O , Deutero, Germany), Milli-Q water (Millipore Advantage A10 system,

Schwalbach, with Millimark Express 40 Filter, Merck, Germany), Lysogeny broth (LB) agar (yeast extract 5 g/L, tryptone 10 g/L, NaCl 5 g/L, Luria/Miller, Carl Roth, Germany) and LB medium (Luria/Miller, Carl Roth, Germany) were purchased as stated. LB agar and LB medium were sterilized at 121°C, 1.2 bar for 15 min in an autoclave (Systec VB-150, Systec GmbH, Linden, Germany).

Preparation of hydrogel and nanofiber substrates

The solutions for preparing CS/PEO nanofiber (NF) and hydrogel (HG) substrates were prepared as reported in the literature.²⁵ Briefly, separate 4 wt.% chitosan solution and 4 wt.% PEO solution were prepared in 0.5 M acetic acid. After stirring for two days, the solutions were mixed in CS:PEO ratio 9:1 by weight and stirred for 5 h. For electrospinning, the solution was filled in a 10 mL syringe. A homebuilt electrospinning set-up³⁹ was used to electrospin CS-PEO nanofibers. The set-up comprised of a syringe pump (Aladdin syringe pump, 941-371-1003, World precision instruments, Florida, USA), a high voltage supply (HCN 35-35000, FuG Elektronik GmbH, Schechen, Germany), a plastic board for mounting of the silicon wafer collectors (P/Boron type OKMETIC, Finland) and a syringe and needle. The electrospun nanofibers were synthesized under ambient conditions. The applied voltage, flow rate and tip-to-collector distance were varied to obtain nanofibers of different fiber diameters. For preparing CS-PEO hydrogels, 5 mL of the mixed solution was poured into a glass petri dish and dried overnight in a laminar flow hood to obtain a thin layer of hydrogel. The nanofiber and hydrogel substrates were neutralized for 5 min with 0.1M KOH while shaking. Afterward, the substrates were rinsed with Milli-Q water.

Modification of CS-PEO hydrogel and nanofibers

EDC/NHS chemistry was used to graft fluorogenic substrate MUG onto the CS-PEO hydrogel and nanofibers, as reported in the literature.¹¹ Briefly, the 1.00 mM MUG solution was prepared in PBS (pH 7.4) followed by the addition of EDC and NHS with 30 mol/mol of -COOH. The solution was stirred for 1 h. The CS-PEO nanofiber and hydrogel substrate were immersed in this modification solution for 12 h while shaking moderately. The modified substrates were then washed with buffered PBS (solution replacement interval: 30 min, for 2 h) until no absorbance of the MUG dye was observed in the washing solutions of both substrates. The samples were then dried overnight under a laminar flow hood.

4.4.2 Methods

ATR-FTIR spectroscopy

ATR-FTIR spectra of the prepared CS-PEO nanofiber and hydrogel substrates were recorded on a Tensor 27 Fourier transform infrared spectrometer (Bruker Optik GmbH, Germany) in the

600–4000 cm^{-1} wavenumber range for the spectra and a spectral resolution of 4 cm^{-1} . The background spectra were obtained by using air.

Scanning electron microscope

Scanning electron microscope analysis was carried out to determine the surface topography of CS/PEO nanofibers and hydrogel substrates. The SEM images were acquired using the CamScan 44 (Applied Beams, Beaverton) electron microscope with a working distance of 27 mm using an acceleration voltage of 25 kV and imaging current of 2.33 A. ImageJ software was used to calculate the average fiber diameter of the NFs.

Nuclear magnetic resonance spectroscopy

A ^1H -NMR spectrum of the modified CS-PEO nanofiber and hydrogel substrates was recorded with a nuclear magnetic resonance (NMR) spectrometer (Bruker Avance 400, Karlsruhe, Germany) using 1% CD_3COOD in D_2O as a solvent.

Fluorescence spectroscopy

For enzymatic kinetic studies, a Varian Cary Eclipse spectrometer (Mulgrave, Victoria, Australia) was used. The spectrum was recorded using 1 cm path-length acrylic cuvettes at 25 °C with a scan rate of 120 nm/min and excitation and emission resolution of 2.5 nm. For *in vitro* bacteria experiments, the fluorescence intensity was measured using a microplate reader (Tecan Infinite M200, Tecan Austria GmbH, Grödig/Salzburg, AUT). The measurements were recorded with fluorescence top reading mode at $\lambda_{\text{ex}} = 365$ nm and $\lambda_{\text{em}} = 450$ nm.

Enzymatic hydrolysis of MUG-modified CS-PEO hydrogel and nanofibers

The enzymatic hydrolysis of the MUG-modified CS-PEO nanofibers and hydrogels was adapted from literature.¹⁵ Briefly, 3.00 mg of dried samples was added in an acrylic cuvette followed by an addition of 1.50 mL of enzyme solutions prepared in PBS (pH 7.4) with varied concentrations. The fluorescence scan was recorded with a Varian Cary Eclipse spectrometer at $\lambda_{\text{ex}} = 365$ nm with 3 min intervals for 4 h after mixing the solution in a cuvette within each measurement scan using a magnetic stirrer. For measurement, the substrate was settled down in the cuvette and the fluorescence of the solution was recorded. The kinetics data were obtained for six different enzyme concentrations (5, 10, 20, 50, 100 and 200 nM) to observe the effect of enzyme concentration on the initial apparent reaction rates. As blank, the modified samples were measured in 1.50 mL PBS (pH 7.40) and the data was subtracted from the samples measured in the presence of enzyme solutions.

Determination of LOD and LOQ

To determine the LOD and LOQ of 4-MU, a standard curve of 4-MU was prepared in PBS (pH 7.40). Different concentrations of 4-MU in PBS were prepared and their fluorescence emission intensities were measured using a Cary fluorescence spectrometer at $\lambda_{\text{ex}} = 365$ nm, scan rate

of 120 nm/min, and a high detector voltage. The obtained emission intensities were plotted against concentration and the data was fitted with linear least square fit. The intercept of the fitted data was considered as background. The LOD and LOQ were calculated as the background added to three and ten times the standard deviation of the background. Afterward, the LOD of enzyme β -Gus in the presence of modified hydrogel and nanofiber substrates was calculated.

Bacteria experiments for β -Gus detection, in vitro

The pathogenic *E. coli* NCTC 10418 here noted as NCTC and non-pathogenic *E. coli* MACH1TM-T1® (Invitrogen, California, USA) here noted as MACH-1 were used in this study. Overnight cultures were prepared by taking single colonies of each bacterial strain in 5.00 mL LB followed by incubating at 37°C for 18 h while shaking at 200 rpm. The OD at 600 nm of each bacteria solution was adjusted to 0.50, corresponding to 10⁸ CFU/mL. For *in vitro* bacteria experiments, 1.00 mg of dried MUG-modified CS-PEO nanofiber and hydrogel substrate were placed in respective wells of a 96-well plate (half area μ -clear well plate (Greiner Bio-One, Germany). 120 μ L of bacterial suspension was added into the wells, the well plate was covered with the clear VIEWsealTM sealer (Greiner Bio-One, Austria) and the well plate was placed in the plate reader. The fluorescence intensity at λ_{ex} = 365 nm and λ_{em} = 450 nm was measured for 24 h. For controls, the fluorescence intensities of the MUG-modified samples were measured in LB medium, and the fluorescence intensities of non-modified samples were also measured in the presence and absence of bacteria suspension. The experiment was done in triplicate.

4.5 References

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5 aPDT effect of encapsulated Ru-based photosensitizers against *Pseudomonas aeruginosa*

This Chapter explores the antimicrobial photodynamic therapy (aPDT) efficacy of Ru-based photosensitizers (PS) against *Pseudomonas aeruginosa* (*P. aeruginosa*). The different PS's encapsulated in PEG₁₁₄-*b*-PLA_x (x= 50, 150, 300, 500) nanocarriers were tested under different irradiation conditions to identify the most effective PEG-*b*-PLA formulation for enhanced aPDT activity against *P. aeruginosa*. Part of the work presented in this chapter has been published in *Pharmaceutics*.[†] The experiments and formal analysis described herein were performed by the author. The Ru-photosensitizers encapsulated in different nanocarriers were provided by Max Müller.

5.1 Introduction and objectives

Antimicrobial resistance (AMR), as outlined in Chapters 1 and 2, represents a major global health challenge. In addition to the development of new and improved diagnostic platforms for bacterial detection discussed in Chapters 3 and 4, it is essential to investigate therapeutic strategies for effective bacterial eradication. Among the ESKAPE pathogens,¹ *P. aeruginosa*, which is also listed as a critical priority 1 pathogen in the WHO's priority list for research and development of new antibiotics,² is one of the most common pathogens causing life-threatening respiratory tract infections, for example in human immunodeficiency virus (HIV)-infected³ and cystic fibrosis (CF)⁴ patients. A big challenge in medicine is the targeted treatment of multidrug-resistant (MDR) and extensively drug-resistant *Pseudomonas aeruginosa* that requires susceptibility screening for the available drug classes, to circumvent resistance mechanisms.⁵ Additionally, tolerance mechanisms due to strong biofilm formation capacity can exacerbate recalcitrant bacterial infections.⁶

To treat MDR bacteria, aPDT has been investigated. Due to its fast and efficient bactericidal effect and its lower risk to built-up of resistance for a variety of different bacterial pathogens,⁷ aPDT was shown to be a promising alternative for the treatment of microbial infections. However, many combinatory approaches for aPDT usually only reach log reduction

[†] Kaur, K.; Müller, M.; Müller, M.; Schönherr, H. Photodynamic eradication of *Pseudomonas aeruginosa* with Ru-photosensitizers encapsulated in enzyme degradable nanocarriers. *Pharmaceutics* **2023**, *15*, 2683.

Author contribution: Conceptualization, H.S. and M.M. (Mareike Müller); Methodology, K.K., M.M. (Max Müller) and M.M. (Mareike Müller); Formal analysis, K.K., M.M. (Max Müller); Investigation, K.K., M.M. (Max Müller); Data curation, K.K., M.M. (Max Müller); Writing- original draft preparation, K.K., M.M. (Max Müller); Writing- review and editing, H.S., M.M. (Mareike Müller); visualization, K.K., M.M. (Max Müller); Supervision, H.S., M.M. (Mareike Müller); Project administration, H.S.; Funding acquisition, H.S., M.M. (Mareike Müller).

factor (LRF) < 3. For instance, methylene blue (MB) loaded electrospun nanofibers showed an LRF of 3⁸, while MB or erythrosine B encapsulated in PCL fibers showed an LRF of only 1.⁹ Inhibitory (not bactericidal) effects were reported for MB in chitosan loaded silver nanoparticles¹⁰, PLA nanofibers loaded with indocynine green and curcumin showed a combined LRF > 4.¹¹ Various studies were carried out to improve the efficiency of aPDT using higher irradiation doses or higher PS concentrations to achieve higher LRF values.^{12,13,14}

Besides this, different metal-based PS have also been investigated for aPDT effect for various applications. In 2022 de Siqueira et al. reviewed phthalocyanines containing aluminium, cobalt, gallium, silicon and zinc as PS in different nanocarriers, such as liposomes, for studying aPDT against bacteria, fungi, viruses and protozoans.¹⁵ However, many known PS used for aPDT show only limited biocompatibility, limited solubility in aqueous or biological media, dark toxicity and reduced photoactivity in the aggregated state, which leads to difficulties in applications.^{16,17}

The use of Ru complexes as PS offers several advantages compared to other PS. Ru(II) based complexes have been extensively studied for PDT effects against fatal cancers and some lead complexes undergo phase II clinical studies for PDT treatment of bladder cancer.^{18,19} Despite this, challenges with the solubility and stability in aqueous media remain to be improved to achieve optimal performance in medical applications.

In this chapter, different Ru-based photosensitisers (PS) were evaluated for their aPDT efficacy against planktonic *P. aeruginosa*. The aPDT effect of PS encapsulated in the PEG₁₁₄-*b*-PLA_x (x = 50, 150, 300, and 500) nanocarriers was also reported. The aPDT parameters for PS-loaded nanocarriers were systematically studied to determine the most efficient conditions for improved aPDT outcomes.

5.2 Results and discussion

5.2.1 Screening of Ru-based photosensitizers

Absorbance measurements

The absorbance spectrum of the Ru-based PS's was recorded to investigate their photophysical properties. The chemical structures of the commercially available Ru(phen)₃ and the various Ru-based PS's synthesized by Prof. Dr. Gilles Lemerrier (Université de Reims Champagne-Ardenne, France) are given in Figure 1. Depending on the partition coefficient, Le Gall et.al reported the lipophilic/hydrophilic character of some of these PS's and observed that the more substituted compounds were more lipophilic.²⁰ MF101 is an amphiphilic complex, MF71 and JM36 are both hydrophilic like the commercially available Ru(phen)₃.

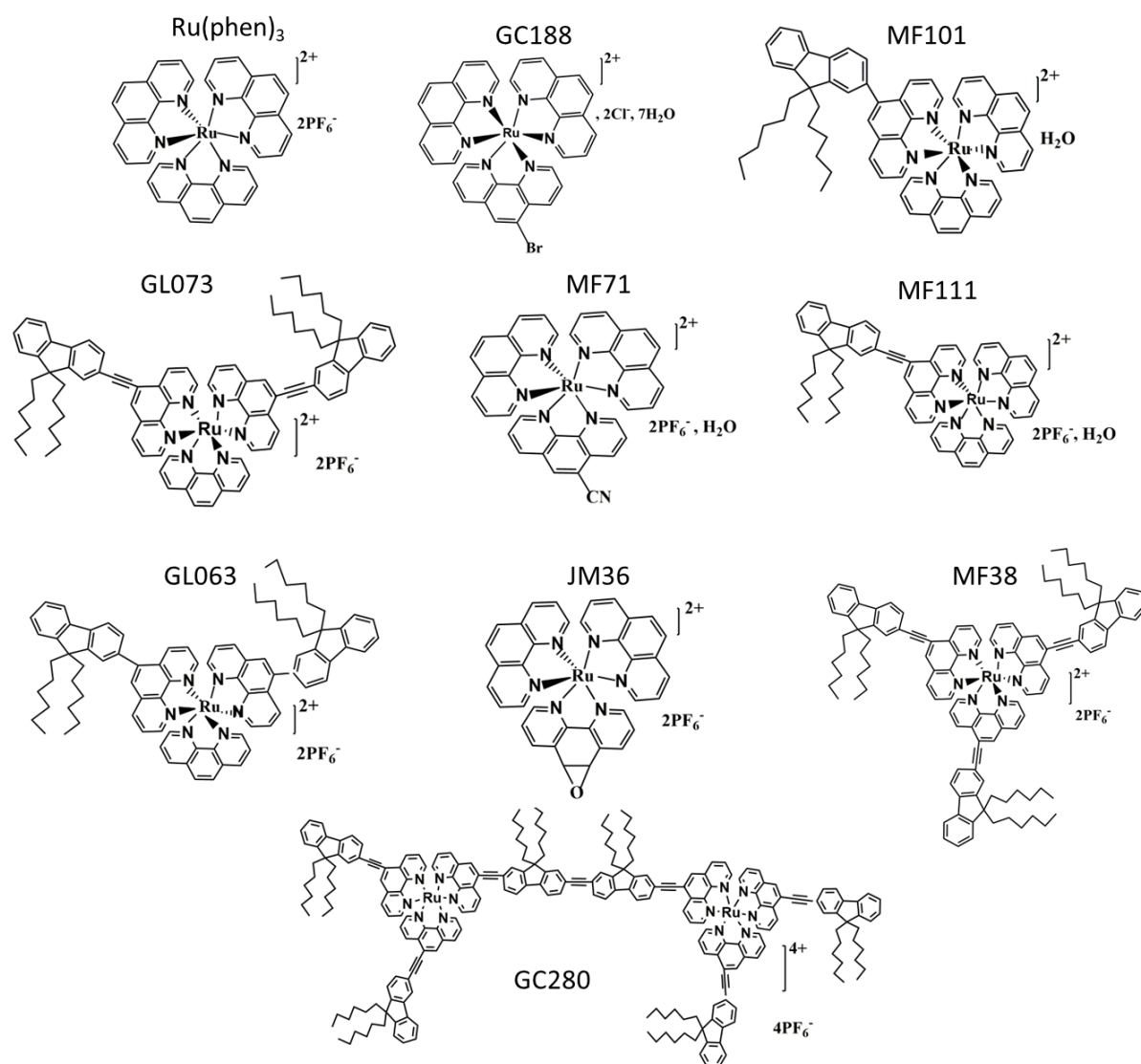


Figure 1: Chemical structures of the different Ru-based photosensitizers investigated for aPDT studies.

UV-Vis measurements were performed using 10 μ M solutions of Ru-based photosensitizers, and the corresponding spectra are given in Figure 2. The obtained spectra were consistent with previously reported data in the literature.²⁰ The UV-Vis spectra were composed of: (i) a strong band in the range of 250-280 nm due to intra-ligand electronic transitions; (ii) a band around 300-400 nm attributed to an intra-ligand charge transfer, and (iii) a broader band centered around 450 nm corresponding to metal-ligand charge transfer (MLCT), which is characteristic of complexes involving polypyridyl ligands. The broad range of MLCT band can be mainly ascribed to vibronic broadenings and/or the overlap of multiple closely spaced electronic transitions.²⁰ Due to the energy and triplet character of the MLCT excited state, these complexes can lead to the efficient production of reactive oxygen species, which is essential for aPDT efficacy.^{7,20}

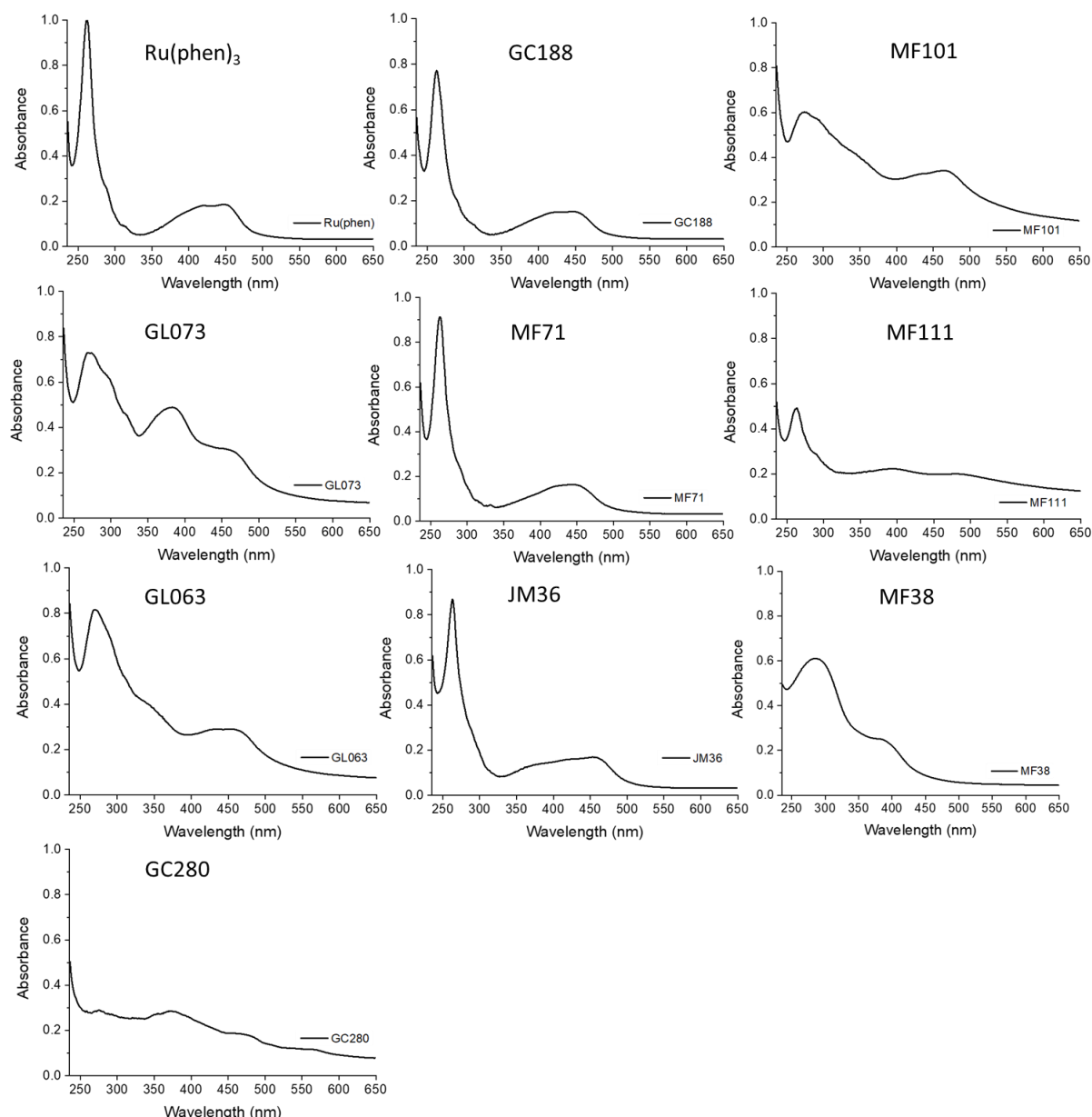


Figure 2: UV-Vis spectra of different Ru-based photosensitizers ([Ru] = 10 μ M). The stock solutions were prepared in DMSO and the desired final solutions were further prepared in water except for MF38 (prepared in tetrahydrofuran).

Detection of singlet oxygen production

Upon photon absorption, the photosensitizers are excited from their ground state to a short-lived singlet excited state, which may undergo intersystem crossing to a longer-lived triplet excited state. From this triplet state, reactive oxygen species, including singlet oxygen are produced through concurrent electron transfer and energy transfer processes.⁷

The production of singlet oxygen species by Ru-based photosensitizers was assessed using the fluorescence intensity of the established commercial SOSG probe.²¹ This detection relies on the reaction of the singlet oxygen produced with the anthracene moiety of the non-fluorescent SOSG probe. In this reaction SOSG is converted to its fluorescent endoperoxide

form (Figure 3). This endoperoxide exhibits fluorescence in the green with an emission maxima at 525 nm (in Tris buffer, 100 mM, pH 7.4) upon excitation at 504 nm.

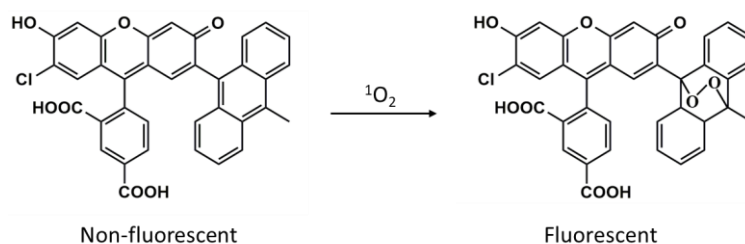


Figure 3: The conversion of the non-fluorescent SOSG probe into its fluorescent endoperoxide form in the presence of singlet oxygen.

Notably, as has been previously reported in the literature, prolonged irradiation of the SOSG probe may lead to an increase in fluorescence intensity, even in the absence of photosensitizers (SOSG in H₂O : MeOH (95: 5) irradiated with 10 ns laser pulses at 355 nm with 20 mJ per pulse, repetition rate 10 Hz).²² This observation has been attributed to the fact that SOSG will sensitize the production of singlet oxygen of the SOSG probe, contributing to the final fluorescence signal.^{22,22}

Figure 4 shows the fluorescence intensity of the SOSG probe observed in the presence of different Ru-based PS's. The fluorescence intensity of the SOSG probe was measured for both irradiated and non-irradiated samples after a defined irradiation time.

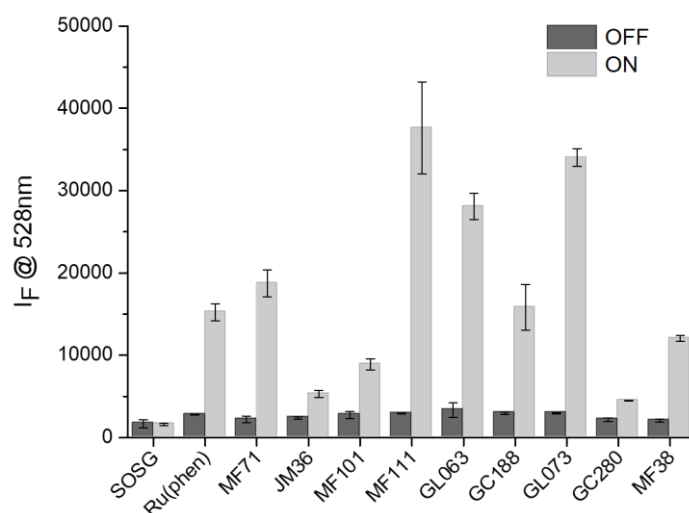


Figure 4: SOSG probe signal observed in experiments with different Ru-based photosensitizers ([Ru] = 10 μ M). The samples were irradiated at λ = 455 nm for 10 min (ON) or were incubated in the dark (OFF). The error bars correspond to the standard deviation of technical replicates (n = 3).

As a control, the neat SOSG probe was also irradiated under exactly the same conditions. No change in fluorescence intensity was observed between the non-irradiated and irradiated SOSG solutions over the monitored time. For non-irradiated Ru-based PS's, slight changes in signal intensity were detected compared to the SOSG probe itself. Whereas, a significant

increase in the probe signal was observed when the Ru-based photosensitizers were irradiated. Following 10 min of irradiation, the fluorescence response of the $^1\text{O}_2$ sensor was ranked in decreasing order as follows:

MF111 > GL073 > GL063 > MF71 > GC188 ~ Ru(phen)₃ > MF38 > MF101 > JM36 > GC280.

The variation in the fluorescence response can be attributed to differences in the amount of $^1\text{O}_2$ generated by the respective PS's. The A higher concentration of detected $^1\text{O}_2$ is essential for effective aPDT to ensure rapid and efficient bacterial eradication. The observed trend in $^1\text{O}_2$ detection indicates that MF111 has the highest potential for aPDT applications, while GC280 is the least effective in facilitating bacterial killing.

aPDT effect on planktonic bacteria

To evaluate the aPDT efficacy of various Ru-based PS, *P. aeruginosa* CF Isolate 5 was selected. The bacterial suspensions prepared in 0.9% saline at a concentration of $3 \times 10^5 \pm 1 \times 10^5$ CFU/mL were treated with PS solutions at final [Ru] of 100 μM and 10 μM , followed by a 15 min incubation. The samples were then subjected to irradiation. To assess bacterial viability, 5 μL aliquots of the treated samples were plated onto LB agar for both non-irradiated and irradiated samples. The corresponding results from the agar spotting method are shown in Figure 5.

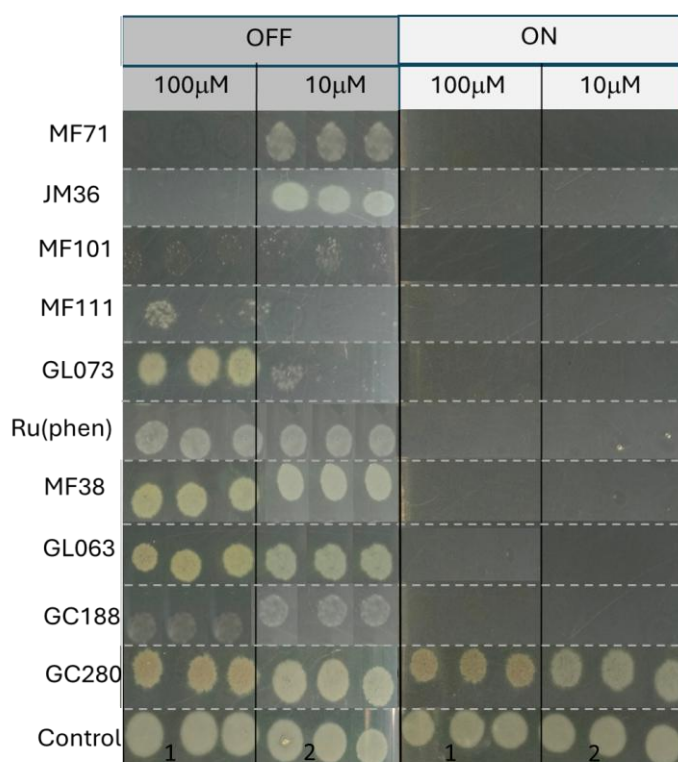


Figure 5: Planktonic aPDT test (agar spot screening) of different Ru-based photosensitizers ([Ru] = 100 μM and 10 μM) against *P. aeruginosa* CF isolate 5. The bacterial suspension ($3 \times 10^5 \pm 1 \times 10^5$ CFU/mL) in 0.9% NaCl was treated with the Ru complexes or as control only with 1% DMSO (control 1), or not treated (control 2: growth control) and incubated for 15 min. The samples were then irradiated for a total 10 min at $\lambda = 455$ nm.

After irradiation, no bacterial growth was observed for any tested photosensitizers for both Ru concentration (100 μ M or 10 μ M), except for GC280. Therefore, an effective aPDT effect was observed for all Ru complexes except GC280. Dark toxicity, defined as cytotoxicity in the absence of irradiation, was observed in some non-irradiated samples. Among the tested photosensitizers, MF71 and JM36, both hydrophilic PS, like the commercially available Ru(phen)₃, exhibited dark toxicity at higher Ru concentrations. MF101, an amphiphilic complex, demonstrated toxicity at both lower and higher concentrations.

GL073, a lipophilic complex, showed toxicity at lower concentration, but not at higher concentration. This is likely due to aggregation of the complex at higher concentrations, which reduces bioavailability and limits its interaction with the bacterial suspension. In contrast, MF111, which is less lipophilic than GL073, showed complete killing at low concentration. At higher concentration, only a few bacterial colonies were observed, attributed to MF111's ability to remain solubilized to some extent and thus available for showing toxicity effects.

The mechanism underlying the dark toxicity of these PS remains unclear, however, solubility is a crucial factor influencing their dark toxicity. Solubility affects the cellular distribution, availability, and molecular interactions of the PS, thereby playing a key role in their toxicity profile in the absence of irradiation. When a PS is well solubilized, it becomes more available to interact with the bacterial suspension, and the extent of these interactions varies depending on the solubilizing capacity of each PS.

These clear aPDT effects observed on agar plates were further studied quantitatively by evaluating the reduction in the concentration of viable bacteria following irradiation. This was achieved by recording the growth kinetics of the treated bacterial suspensions through OD measurements at 600 nm. Monitoring growth kinetics enabled the estimation of the reduction in bacterial density. Figure 6 shows the growth kinetics curves for both non-irradiated and irradiated bacterial suspensions treated with different Ru-based photosensitizers at a final [Ru] of 10 μ M.

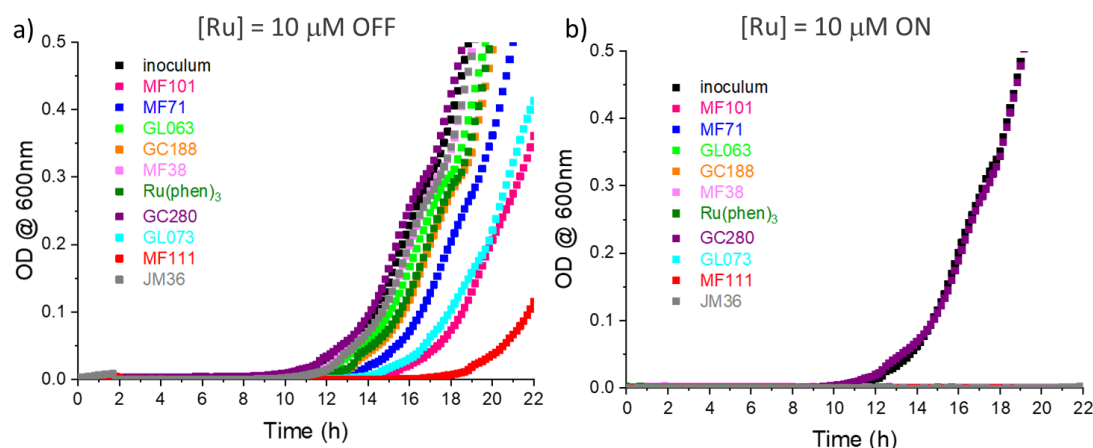


Figure 6: Growth kinetics curves of *P. aeruginosa* CF isolate 5 in the presence of different Ru-based photosensitizers ([Ru] = 10 μM) for samples a) kept in the dark, and b) irradiated samples for an accumulated 10 min irradiation at $\lambda = 455$ nm.

For non-irradiated samples (Figure 6a), the growth kinetics of bacteria treated with Ru complexes at a final [Ru] = 10 μM were expected to align with that of bacterial inoculum. However, some Ru complexes induced varying delays in bacterial growth, indicating differing levels of dark toxicity as discussed above (for Figure 5). Upon irradiation (Figure 6b), no bacterial growth was observed in samples treated with Ru complexes, except for GC280, which exhibited growth kinetics comparable to the bacterial inoculum.

Similarly, Figure 7 shows the growth kinetics curves of non-irradiated and irradiated bacterial suspensions treated with different Ru-based photosensitizers at a final [Ru] = 100 μM.

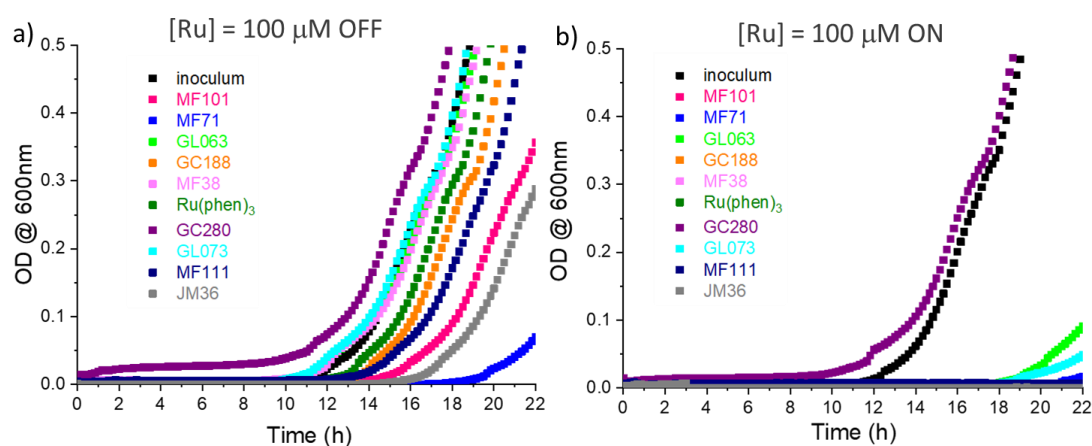


Figure 7: Growth kinetics curves of *P. aeruginosa* CF isolate 5 in the presence of different Ru-based photosensitizers ([Ru] = 100 μM) for samples a) kept in the dark, and b) irradiated samples for an accumulated 10 min irradiation at $\lambda = 455$ nm.

In the presence of 100 μM Ru complexes, delays in the bacterial growth kinetics were also observed for non-irradiated samples, indicating potential dark toxicity effects. After irradiation, bacterial growth was observed in the case of GC280, similar to the growth control. For GL063,

and GL073, delayed bacterial growth was observed after 18 h incubation, suggesting the presence of very few bacterial colonies after irradiation. For rest of the complexes, no bacterial curves were observed, suggesting complete killing. This observation is consistent with the observation obtained from spotting method (Figure 5).

The growth kinetics data were further utilized to estimate the concentration of viable bacteria. A standard curve was obtained using serial dilutions (10-fold) of the bacterial inoculum (Figure 8). The time required to reach OD_{600nm} of 0.1 was recorded for each dilution and plotted against the corresponding bacterial dilution.

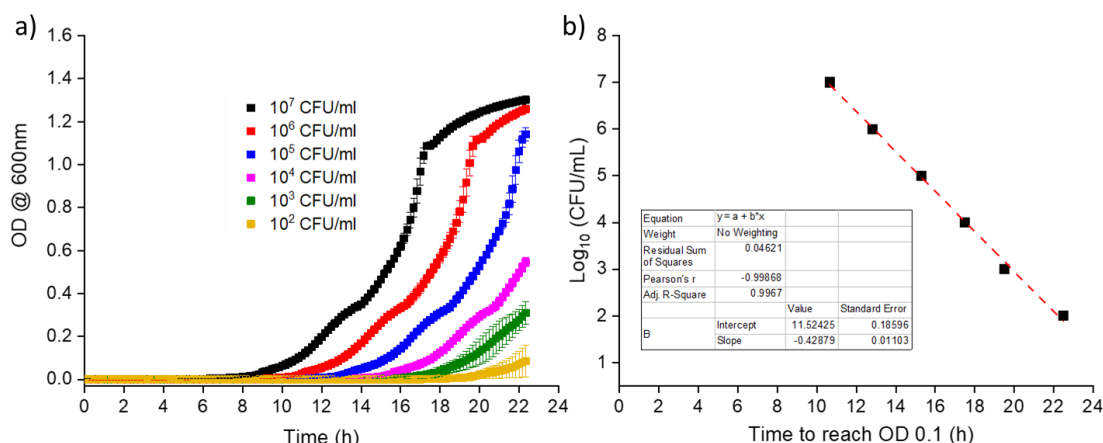


Figure 8: a) Growth kinetics curves of serial dilutions of *P. aeruginosa* CF isolate 5 in LB medium. b) Standard curve for the estimation of viable bacteria obtained from growth kinetics curves. The error bars correspond to the standard deviation of technical replicates ($n = 3$).

This standard curve was used to estimate the viable bacterial concentrations in suspensions treated with various Ru-based photosensitizers. The log₁₀ CFU/mL for both non-irradiated and irradiated samples treated with different Ru-based photosensitizers were then calculated and shown in Figure 9.

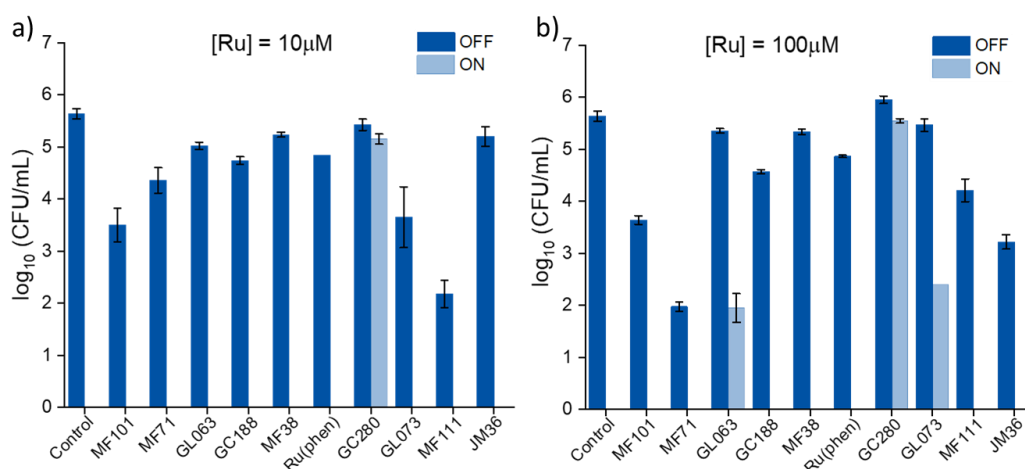


Figure 9: The log CFU/mL of the non-irradiated (OFF) and irradiated (ON) Ru-based photosensitizers in the presence of *P. aeruginosa* CF isolate 5 for Ru concentrations a) 10 μM and b) 100 μM. The values were calculated using the growth kinetics curves shown in Figures 5 and 6. The control is the bacterial inoculum. The absence of

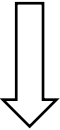
aPDT effect of encapsulated Ru-based photosensitizers against *Pseudomonas aeruginosa*

bar corresponds to no bacterial colonies. The error bars correspond to the standard deviation of technical replicates ($n = 3$).

At both Ru concentrations, different CFU/mL values were observed under non-irradiated conditions. Notably, only MF111 at 10 μ M and MF71 at 100 μ M demonstrated a bactericidal effect (≥ 3 log reduction factor (LRF)) already in the dark. To lower/diminish the dark toxicity effects of PS's in non-irradiated conditions, lower concentrations of photosensitizers should be used. All other Ru complexes, except the ineffective GC280, showed a bactericidal effect exclusively after irradiation.

To correlate the relation of $^1\text{O}_2$ detection and the observed aPDT effects for different PS's, the aPDT efficacy of different PS's at $[\text{Ru}] = 10 \mu\text{M}$ (Figure 9a) was compared with their SOSG probe signals for $^1\text{O}_2$ detection (Figure 4). The decreasing order of the SOSG probe signal and aPDT effect were compiled in Table 1, with complexes showing similar values grouped.

Table 1: The decreasing order of the SOSG endoperoxide emission intensity and aPDT effect observed with 10 μ M Ru-based photosensitizers following 10 min irradiation.

	<i>SOSG signal</i>	<i>aPDT effect</i>
<i>Decreasing order of $^1\text{O}_2$ detection and aPDT effect with 10 μM Ru complexes</i> 	MF111	MF111 MF101 GL073 MF71 GC188 Ru(phen) ₃ GL063 JM36 MF38
	GL073	
	GL063	
	MF71	
	GC188	
	Ru(phen) ₃	
	MF38	
	MF101	
	JM36	
	GC280	GC280

Upon irradiation, a significant increase in SOSG signals was observed, except for GC280. MF111 complex showed the highest signal intensities. In the decreasing order for both the SOSG signal and aPDT results, GC280 is the one with a lower SOSG signal and shows no aPDT effect. However, for other photosensitizers, no clear correlation could be established between the SOSG probe signals and the aPDT outcomes due to complete killing after irradiation. This indicates that the SOSG signal may not correlate linearly with the aPDT

outcomes either because sufficient $^1\text{O}_2$ is already produced by PS's to achieve maximal efficacy, or due to the possible involvement of other reactive oxygen species contributing to the overall aPDT effect. To identify a more discernible trend, the aPDT effect should be evaluated at Ru concentrations lower than $10\mu\text{M}$.

5.2.2 Impact of parameter variation on SOSG signal

SOSG is widely used to detect $^1\text{O}_2$, but its response can be influenced by various factors such as PS concentration, medium, probe concentration, irradiation time, and oxygen availability. Studying these parameters helps to validate that the SOSG signal reflects true $^1\text{O}_2$ levels. So the various factors that were further investigated include the effect of the medium of the photosensitizer solution, the concentration of the photosensitizer and SOSG probe, and the addition of fresh SOSG after the probe signal had reached a plateau.

Effect of changing medium

The effect of the medium on singlet oxygen production was investigated using solutions of the commercially available $\text{Ru}(\text{phen})_3$ in different media (Figure 10). The samples were irradiated at 5 min ON-5 min OFF intervals over a total duration of 30 min.

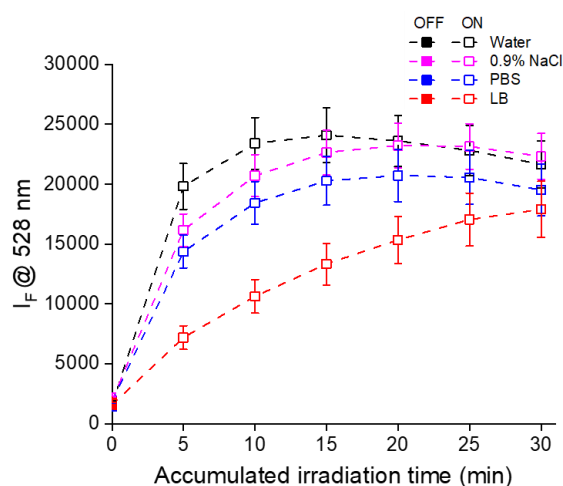


Figure 10: SOSG probe signal for detecting singlet oxygen production by $25\mu\text{M}$ $\text{Ru}(\text{phen})_3$ prepared in different solutions. The samples were irradiated at $\lambda = 455\text{ nm}$ with 5 min intervals for 30 min. The error bars correspond to the standard deviation of technical replicates ($n = 3$).

With increasing irradiation time, a significant increase in the SOSG signal was observed during the initial 15 min. However, the increase was less pronounced in the following order of media:

$$\text{water} < 0.9\% \text{ saline} < \text{PBS} < \text{LB}$$

This trend can be attributed to differences in viscosity and ionic strength of the media. Water, with the lowest viscosity and nearly zero ionic strength, allows unhindered diffusion of $^1\text{O}_2$ and causes minimal interference with SOSG fluorescence. As viscosity and ionic strength increase from water to saline, PBS and LB, the diffusion of $^1\text{O}_2$ becomes increasingly restricted. LB has the highest viscosity, which significantly hinders the $^1\text{O}_2$

diffusion. Additionally, its rich organic contents may quench either $^1\text{O}_2$ directly or the fluorescence of SOSG probe thereby reducing the overall signal.

After 5 min irradiation, the signal intensity was ~ 4 times higher in the presence of water and ~ 3 times higher in saline and PBS compared to LB. This suggests that the medium composition influences the generation or detection of singlet oxygen. After a certain irradiation time, the SOSG probe signal did not increase further and plateaued. This plateau could be attributed to several possible factors: (i) depletion of the photosensitizer, limiting further $^1\text{O}_2$ production; (ii) depletion of the SOSG probe, reducing its capacity to detect additional $^1\text{O}_2$; or (iii) depletion of available oxygen in the medium, which is essential for $^1\text{O}_2$ generation. These factors highlight the need to consider medium properties and reactant availability when studying photosensitizer efficiency.²³

Effect of concentration of Ru(phen)₃ and SOSG

The effect of varying the concentrations of Ru(phen)₃ and SOSG on the probe signal was then systematically studied over an accumulated irradiation period of 30 min with 5 min ON-5 min OFF interval (Figure 11).

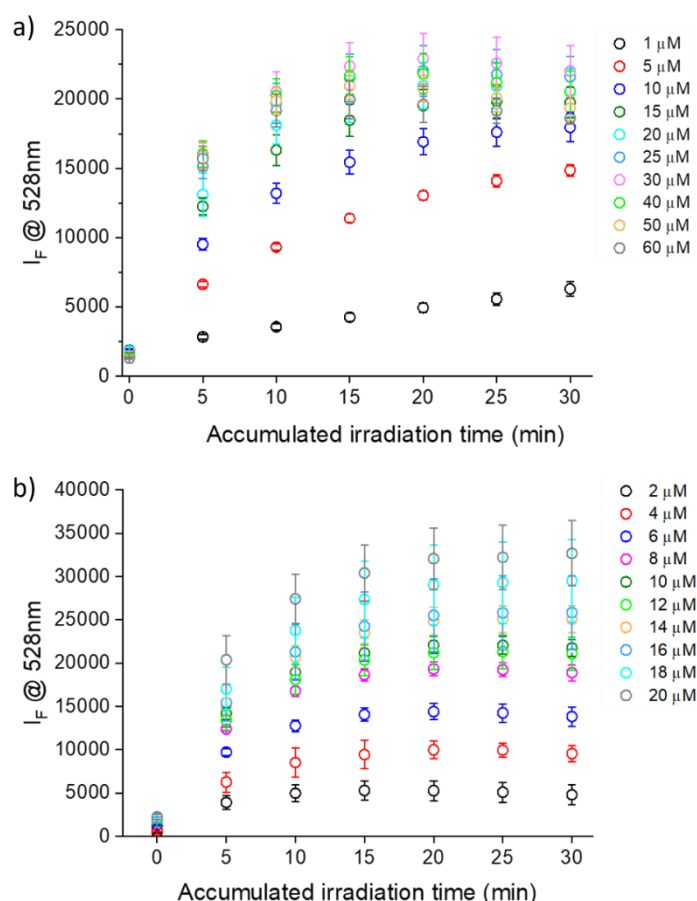


Figure 11: SOSG probe signal for detecting singlet oxygen production a) varied Ru(phen)₃ concentrations with constant SOSG (10 μM final concentration), b) varied final SOSG concentration with constant Ru(phen)₃ (20 μM final concentration). The samples were irradiated at $\lambda = 455$ nm with 5 min intervals for 30 min. The error bars correspond to the standard deviation of technical replicates ($n = 3$).

The effect of varying Ru(phen)₃ concentration on the probe signal was studied by varying Ru(phen)₃ concentration from 1 μ M to 60 μ M, while maintaining a fixed SOSG concentration of 10 μ M in the solution (Figure 11a). The probe signal intensity increased with increasing Ru(phen)₃ concentration and longer irradiation durations. However, beyond 20 μ M Ru(phen)₃, no significant enhancement in the probe signal was observed despite further increases in Ru(phen)₃ concentration. This can be attributed to multiple factors. First, the generation of singlet oxygen depends on the availability of molecular oxygen as a substrate. If the oxygen concentration becomes limited within the system, further increases in photosensitizer concentration do not translate to additional ¹O₂ production. Second, the SOSG probe has a finite number of reactive sites that interact with singlet oxygen. Once these sites are fully consumed, an increase in the ¹O₂ concentration (from more photosensitizer) does not result in higher fluorescence signals. Additionally, the aggregation of the photosensitizer at high concentrations could also reduce its capacity to absorb light and efficiently transfer energy to molecular oxygen, further reducing ¹O₂ production.

As for >20 μ M [Ru(phen)₃], not much change in the SOSG signal was observed so this PS concentration was chosen to study the effect of SOSG concentration on the detection signal. Figure 11b shows the SOSG probe signal response at varying SOSG concentrations, at a constant Ru(phen)₃ concentration of 20 μ M. The probe signal intensity increases with increasing probe concentration and the SOSG signal was plateaued after 15 min in all cases. If compared after 5 min irradiation, the probe signal increased linearly until 10 μ M SOSG, and at higher probe concentrations the increase in signal was not very significant. A possible explanation can be that at a constant photosensitizer concentration, the amount of singlet oxygen produced is limited. Even if more probe molecules are added, the available singlet oxygen may become insufficient to react with all the probe molecules. Once the probe concentration exceeds the amount of singlet oxygen produced, additional probe molecules remain unreacted, and the fluorescence signal levels off.

Another important factor to consider while studying SOSG probe signal is the short lifetime (~ 4 μ s in water^{24,25}) and limited diffusion range (~ 125 nm in water²⁶) of singlet oxygen. These properties restrict its ability to interact with distant probe molecules before decaying. At higher probe concentrations, a substantial fraction of the probe may lie beyond the effective range ¹O₂, limiting the fluorescence signal increase. Therefore, optimizing the probe concentration is critical to maximizing sensitivity and avoiding diminishing returns. Also it is well reported that for longer irradiation times SOSG and ¹O₂ can be converted to other reactive species, which interfere with the signal response.^{21,22}

Effect of adding SOSG after reaching a plateau

In all SOSG experiments, a plateau in the SOSG signal was observed. To understand the effect of adding fresh SOSG solution after reaching this plateau, 25 μM of Ru(phen)₃ (a hydrophilic PS) and MF38 (a hydrophobic PS) were mixed with SOSG (final [SOSG] = 10 μM) and irradiated. Fresh SOSG solution was added twice at 20 min intervals during irradiation. Following the first addition, the final [SOSG] in the solutions was 18 μM and 25 μM after the second addition. The resulting SOSG signal intensity over accumulated time is shown in Figure 12.

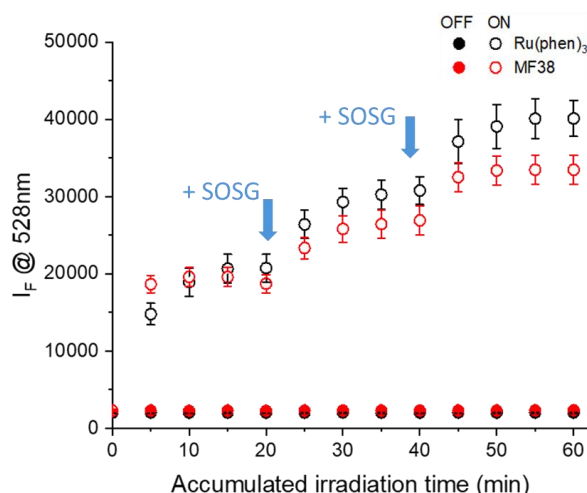


Figure 12: SOSG probe signal of neat 25 μM neat Ru(phen)₃ and MF38 over accumulated irradiation time. 10 μl of 100 μM SOSG was added after every 20 min. The error bars correspond to the standard deviation of technical replicates ($n = 3$). The samples were irradiated at $\lambda = 455$ nm.

It was observed that after reaching a plateau, the addition of fresh SOSG resulted in only a slight increase in probe signal, which subsequently reached a new plateau. Since this trend was consistent for both the hydrophilic and hydrophobic PS, it is unlikely to be due to PS aggregation. Among other effects the SOSG may be depleted here and the possible explanation for the slight increase in signal after each addition of SOSG could be the introduction of additional oxygen and freshly added SOSG during the mixing step.

It can be concluded that careful consideration of all experimental parameters influencing SOSG signal is essential to ensure accurate quantification of $^1\text{O}_2$ production. Failure to account these parameters may lead to signal artifacts, underestimation or overestimation of $^1\text{O}_2$ levels.

5.2.3 Detection of $^1\text{O}_2$ in PS-loaded polymeric nanocarriers

Before evaluating the aPDT efficacy of PS-loaded PEG₁₁₄-*b*-PLA_x ($x = 50, 150, 300, 500$) nanocarriers, it is critical to assess their singlet oxygen production. This section includes the investigation of the hypothesis of enzymatically triggered release of PS from the nanocarriers,

$^1\text{O}_2$ production was measured from both hydrophobic and hydrophilic PS encapsulated in nanocarriers before and after enzymatic treatment.

Effect of enzymatic treatment of PS-loaded nanocarriers with Proteinase K

The enzyme labile polymer PLA within the block copolymer facilitates the on-demand release of PS in the presence of proteinases. The proteinases produced by *P. aeruginosa* hydrolyze peptide and ester bonds. Utilizing Proteinase K, a model enzyme known to destabilize micelles and polymersomes containing PLA polymers, facilitates the enzymatically triggered release of PS.^{27,28} It has been hypothesized that this release mechanism allows the liberated PS to diffuse towards the targeted bacteria, potentially enhancing the aPDT efficacy. To investigate this hypothesis, the SOSG probe was used to detect the singlet oxygen generated by encapsulated PS before and after enzymatic treatment. Hydrophilic Ru(phen)₃ and hydrophobic MF38 were encapsulated into PEG₁₁₄-*b*-PLA₅₀ micelles with estimated PS concentrations of ~10-13 μM for MF38 and ~ 33 μM for Ru(phen)₃ in suspension. The encapsulated samples were mixed in a 1:1 ratio with 10mg/mL Proteinase K solution and incubated for 20 h. Afterward, the singlet oxygen production was detected using the SOSG probe (Figure 13) before and after enzymatic treatment. The samples were irradiated for an accumulated time of 30 min with 5 min intervals.

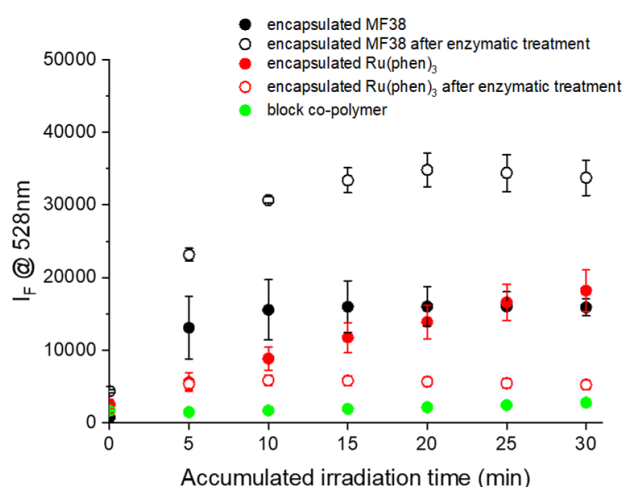


Figure 13: SOSG probe signal as a function of accumulated irradiation time for encapsulated MF38 and Ru(phen)₃ in PEG₁₁₄-*b*-PLA₅₀ micelles treated with and without the enzyme Proteinase K. The error bars correspond to the standard deviation of technical replicates ($n = 3$). The samples were irradiated at $\lambda = 455$ nm for an accumulated time of 30 min with 5 min ON-5min OFF intervals.

No increase in signal was observed for unloaded micelles confirming the absence of singlet oxygen production. In contrast, in both encapsulated Ru(phen)₃ and MF38 the SOSG probe signal increased over time, signaling the detection of singlet oxygen during irradiation. After enzymatic treatment, a higher signal increase was observed for MF38 compared to its encapsulated form. This enhancement is attributed to the release of MF38, a lipophilic PS

encapsulated within the micelle core, upon exposure to Proteinase K. Thereby facilitating more efficient singlet oxygen production.

For encapsulated Ru(phen)₃, a decrease in probe signal was observed after enzymatic treatment. To interpret this, it is important to consider the localization of PS within polymeric micelle suspension before and after enzymatic degradation. As a hydrophilic molecule, Ru(phen)₃ is likely located in the hydrophilic corona of the micelles. Therefore, enzymatic treatment does not result in additional release of PS into the surrounding medium. The observed decrease in signal intensity may be due to the reduced oxygen availability following micelle degradation or the slower diffusion of singlet oxygen to the SOSG probe.

Due to the limited availability of MF38 complex, the aPDT experiments were conducted using polymeric nanocarriers encapsulating commercially available Ru(phen)₃.

5.2.4 Investigation of aPDT parameters with Ru(phen)₃-loaded nanocarriers

The parameters for aPDT using Ru(phen)₃-loaded nanocarriers were investigated in the presence of planktonic bacteria. Firstly, the influence of the medium used to prepare the bacterial suspensions and irradiation times was studied. Subsequently, the Ru(phen)₃-loaded nanocarriers were treated with the enzyme Proteinase K and then tested for aPDT efficacy (*Test 1*). In the next step, the Ru(phen)₃-loaded nanocarriers were enzymatically treated with Proteinase K. To investigate if the Ru(phen)₃ is located with the degraded polymeric nanocarriers or in the supernatant, the samples were centrifuged to separate pellets and supernatants. The pellets were then resuspended in water. For aPDT evaluation, three experimental conditions were tested (i) encapsulated PS, (ii) resuspended pellet, and (iii) supernatant obtained after enzymatic treatment of the PS-loaded nanocarriers (*Test 2*).

Effect of the medium of bacteria suspension on aPDT efficacy

To investigate the effect of media used to prepare bacterial suspension on aPDT efficacy *Pseudomonas aeruginosa* lab strain ATCC was used. The bacterial suspensions were prepared by centrifuging overnight cultures of PA ATCC to obtain pellets, which were then resuspended in either LB medium or 0.9% saline. For aPDT experiments, the PS-loaded nanocarriers were mixed with the different media in 1:1 ratio resulting in final concentrations to be ~ half of the undiluted PS-loaded nanocarriers. The loaded nanocarriers (estimated [Ru(phen)₃] in the suspensions listed in Table 2) were added to the different bacteria suspensions and subjected to irradiation following incubation for 15 min.

Table 2: Concentration of the encapsulated Ru(phen)₃ in suspensions of polymeric assemblies.

	Assemblies	Concentration in suspension (μM)	Concentration after mixing with bacteria suspension (μM)
PEG ₁₁₄ - <i>b</i> -PLA ₅₀₀	Vesicles	16.1 $\pm$ 0.2	8.1 $\pm$ 0.1
PEG ₁₁₄ - <i>b</i> -PLA ₃₀₀	Vesicles	32.1 $\pm$ 5.1	16.0 $\pm$ 2.5
PEG ₁₁₄ - <i>b</i> -PLA ₁₅₀	Micelles	47.2 $\pm$ 3.2	23.6 $\pm$ 1.6
PEG ₁₁₄ - <i>b</i> -PLA ₅₀	Micelles	33.6 $\pm$ 1.7	16.8 $\pm$ 0.8

The irradiation was performed with 5 min ON-5min OFF intervals for accumulated irradiation times of 10 and 15 min. As a control, the aPDT efficacy of neat Ru(phen)₃ at a final concentration of 25 μM was also investigated. Figure 14 shows the photographs of spotted bacterial colonies of PA ATCC in the presence of neat Ru(phen)₃ without and with irradiation.

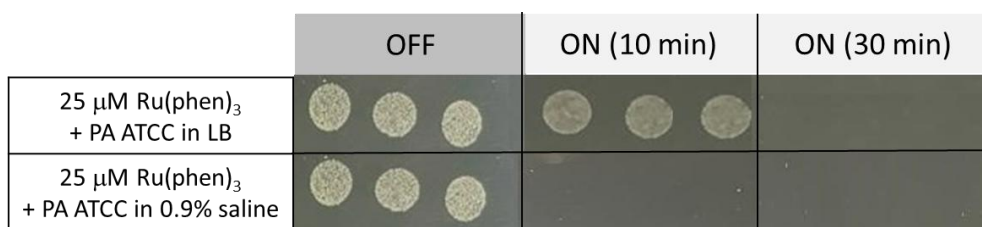


Figure 14: Photographs of spotted bacterial colonies (after growth for 18 h) of PA ATCC suspensions in LB and 0.9% saline without and with irradiation at $\lambda = 455$ nm of neat Ru(phen)₃. The samples were irradiated for 10 min and 30 min (ON) at $\lambda = 455$ nm or incubated in the dark for 30 min (OFF).

For bacterial suspension prepared in LB medium, bacterial growth was observed for samples irradiated for 10 min. However, no colonies were observed when the total irradiation time was increased to 30 min, while for the bacteria suspension prepared in 0.9% saline, no bacterial colonies were observed even after 10 min of total irradiation.

Figure 15 shows the photographs of spotted bacterial colonies of PA ATCC in the presence of Ru(phen)₃-loaded polymeric assemblies for non-irradiated and irradiated samples incubated with bacteria suspensions prepared in LB medium.

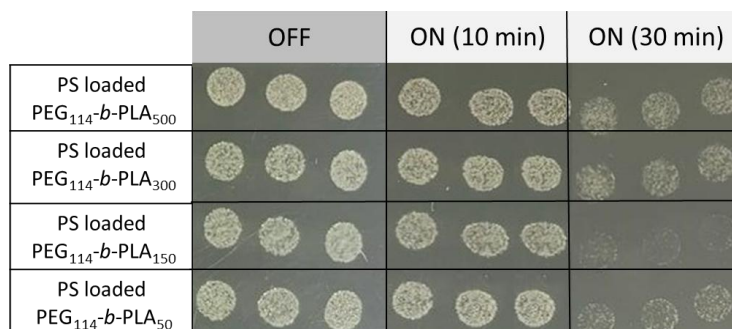


Figure 15: Photographs of spotted bacterial colonies (after growth for 18 h) of PA ATCC suspension in LB without and with irradiation at $\lambda = 455$ nm of Ru(phen)₃-loaded polymer assemblies. The samples were irradiated for 10 and 30 min (ON) at $\lambda = 455$ nm or incubated in the dark for 30 min (OFF).

aPDT effect of encapsulated Ru-based photosensitizers against *Pseudomonas aeruginosa*

No aPDT effect was observed with encapsulated Ru(phen)₃ in the different polymeric assemblies following 10 min irradiation. As the irradiation time was increased to 30 min, a slight reduction in bacterial colonies was observed in all samples. On comparing Ru(phen)₃ concentrations in different nanocarrier suspensions (Table 2), PEG₁₁₄-*b*-PLA₁₅₀ is the one with a higher PS concentration in the suspension, which correlated to the noticeable decrease in colony growth after 30 min irradiation.

Additionally, the spotting results for the bacterial colonies in the case of bacteria suspension prepared in 0.9% saline are shown in Figure 16.

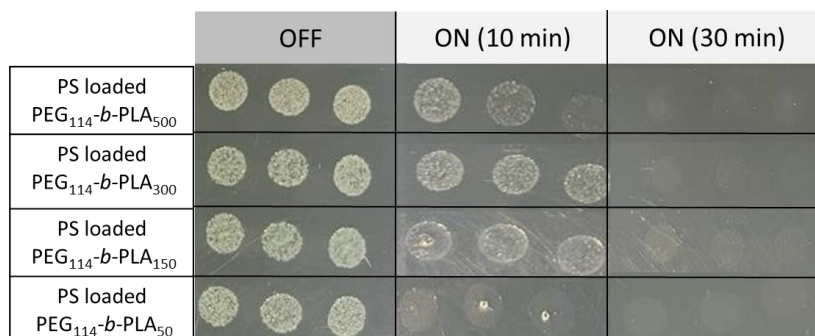


Figure 16: Photographs of spotted bacterial colonies (after 18 h growth) of PA ATCC suspension in 0.9% saline without and with irradiation at $\lambda = 455$ nm of Ru(phen)₃-loaded polymer assemblies. The samples were irradiated for 10 and 30 min (ON) at $\lambda = 455$ nm or incubated in the dark for 30 min (OFF).

Compared to non-irradiated samples, reduced bacterial colony growth was observed even after 10 min irradiation. This aPDT effect was further enhanced with increased irradiation time.

The greater aPDT efficacy observed in the bacterial suspension prepared in saline compared to those in the LB medium can be attributed to several factors. The LB medium is nutritionally rich medium (containing tryptone, yeast extract, and saline) which can quench singlet oxygen generated during aPDT, whereas saline lacks such components resulting in more effective ¹O₂ activity. This is supported by Figure 10, which shows a lower SOSG probe signal in the LB medium compared to saline. Additional factors that may contribute to the observed differences include variations in osmotic pressure between media, which can influence bacterial membrane permeability and subsequently affect photosensitizer uptake. Furthermore, the composition of the medium can alter bacterial physiology, leading to differences in the uptake of molecules by the bacteria.

Therefore, when the bacterial suspensions were prepared in saline, more efficient aPDT results can be achieved with Ru(phen)₃-loaded nanocarriers by increasing the irradiation time. Consequently, further experiments were done using bacterial suspensions prepared in 0.9% saline.

aPDT effects after the enzymatic treatment of Ru(phen)₃-loaded nanocarriers (Test 1)

To investigate the hypothesis that enzymatically triggered photosensitizer release improves aPDT outcomes, the Ru(phen)₃-loaded nanocarriers (concentrations listed in Table 2) were mixed at a 1:1 ratio with 10 mg/mL Proteinase K and incubated for 20 h. Proteinase K can hydrolyze peptide and ester bonds, including those in PLA polymers. Figure 17 shows pictures samples containing different PLA chain lengths following enzymatic treatment.

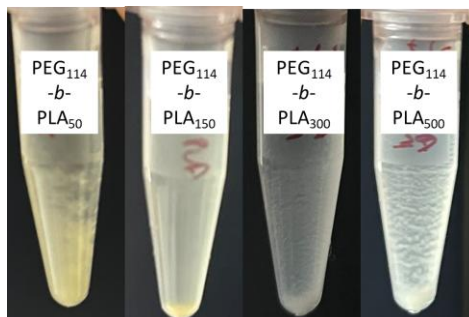


Figure 17: Pictures of the Ru(phen)₃-loaded polymeric assemblies after the 20 h incubation with the Proteinase K.

After 20 h of incubation with Proteinase K, sedimentation of the polymers and the photosensitizer was observed. This degradation is attributed to the enzymatic cleavage of PLA repeating units, which leads to the release of photosensitizer, followed by disintegration of the nanocarrier and finally cleavage into the formation of lactic acid.²⁸ For aPDT experiments, the samples after enzymatic action were vortexed and then mixed with bacterial suspension prepared in 0.9% saline. After 15 min incubation, the samples were irradiated for 10 and 30 min. Figure 18 shows photographs of the spotted bacterial colonies of PA ATCC treated with PS-loaded nanocarriers after enzymatic treatment.

		OFF	ON (10 min)	ON (30 min)
After enzymatic treatment	PS loaded PEG ₁₁₄ -b-PLA ₅₀₀			
	PS loaded PEG ₁₁₄ -b-PLA ₃₀₀			
	PS loaded PEG ₁₁₄ -b-PLA ₁₅₀			
	PS loaded PEG ₁₁₄ -b-PLA ₅₀			

Figure 18: Photographs of spotted bacterial colonies (after 18 h growth) of PA ATCC suspension in 0.9% saline without and with irradiation at $\lambda = 455$ nm of Ru(phen)₃-loaded polymer assemblies after enzymatic treatment for 20 h. The samples were irradiated for 10 and 30 min (ON) at $\lambda = 455$ nm or incubated in the dark for 30 min (OFF).

After 10 min irradiation, no significant difference in bacterial colony growth was observed compared to non-irradiated samples. However, a noticeable reduction in bacterial colonies was observed only after 30 min of irradiation, with more pronounced aPDT effects for Ru(phen)₃-loaded in PEG₁₁₄-b-PLA₁₅₀ and PEG₁₁₄-b-PLA₅₀ samples, attributed to higher Ru(phen)₃ concentrations in the suspensions of these assemblies (Table 2).

Comparing the spotting results after 30 min irradiation for Ru(phen)₃-loaded nanocarriers without enzymatic treatment (Figure 16) and with enzymatic treatment (Figure 18), no visible bacterial colonies were observed with the samples without enzymatic treatment. Therefore, the hypothesis that enzymatic treatment would enhance aPDT efficacy was not supported by the experimental results.

aPDT effect after enzymatic treatment of Ru(phen)₃-loaded nanocarriers (Test 2)

To investigate if the Ru(phen)₃ is located with the degraded polymeric nanocarriers or in the supernatant, the samples were centrifuged to separate pellets and supernatants. For this experiment, the Ru(phen)₃-loaded nanocarriers with concentrations given in Table 3 were used. The loaded nanocarriers were mixed in a 1:1 ratio with the bacterial suspension to have their final concentrations be ~ half of the undiluted loaded nanocarriers. PA Isolate 5 suspension was prepared in 0.9% saline for the experiments.

Table 3: Concentration of the Ru(phen)₃ in the suspensions.

	Assemblies	Concentration in suspension (μM)	Concentration after mixing with bacteria suspension (μM)
PEG ₁₁₄ - <i>b</i> -PLA ₅₀	Vesicles	11.3 ± 0.3	5.65 ± 0.15
PEG ₁₁₄ - <i>b</i> -PLA ₁₅₀	Vesicles	54.0 ± 1.8	27 ± 0.9
PEG ₁₁₄ - <i>b</i> -PLA ₃₀₀	Micelles	6.0 ± 0.4	3.0 ± 0.2
PEG ₁₁₄ - <i>b</i> -PLA ₅₀₀	Micelles	15.5 ± 0.2	7.75 ± 0.1

For enzymatic treatment, the Ru(phen)₃-loaded polymeric nanocarriers were mixed in 1:1 ratio with 5 mg/mL Proteinase K and incubated for 18h. The samples were then centrifuged at 10,000 rpm for 2 min to form pellets. Figure 19 shows images of the collected pellets.

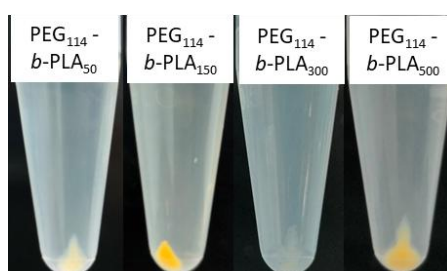


Figure 19: Pictures of collected pellets of the Ru(phen)₃-loaded nanocarriers after their enzymatic treatment for 18 h followed by centrifugation.

The supernatant was separated, and the pellets were resuspended in water. The following samples were then investigated for the aPDT experiment: Ru(phen)₃ loaded nanocarriers (referred to as *PS-loaded*), a resuspended pellet after enzymatic treatment (referred to as *pellet*), and the supernatant collected after enzymatic treatment (referred to as *supernatant*).

These samples were mixed with the bacterial suspension and incubated for 15 min in the dark. Afterward, the light irradiation with 5 min ON -5min OFF intervals was performed for a

total of 10 and 30 min. As a control experiment, 25 μM of neat $\text{Ru}(\text{phen})_3$ was tested against PA Isolate 5. Figure 20 shows the SOSG probe signals and $\log_{10}$ CFU/mL plots for the control samples after 10 min irradiation. As expected, after irradiation no change in probe signal was observed in the absence of PS (Figure 20a). A higher signal was observed for PS and PS mixed with bacterial suspension after the irradiation.

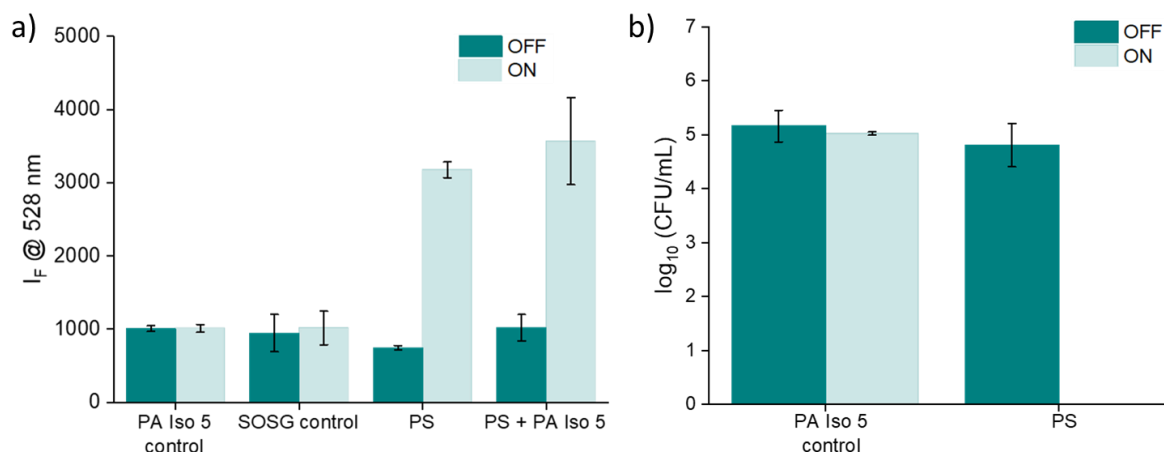


Figure 20: a) SOSG probe signal of the control samples, b) $\log_{10}$ CFU/mL bar plots of the 25 μM $\text{Ru}(\text{phen})_3$ tested in the presence of PA Isolate 5 suspension. No bar corresponds to no countable colonies. OFF: non-irradiated samples, ON: samples after 10 min irradiation at $\lambda = 455 \text{ nm}$.

Figure 20b shows the $\log_{10}$ CFU/mL of the neat PS tested against PA Isolate 5. After 10 min irradiation, no change in colony forming unit was observed for bacterial control. Whereas no countable colonies were detected in the presence of PS.

Figure 21 shows the bar plots of the $\log_{10}$ CFU/mL for the PS-loaded nanocarriers, the resuspended pellets, and the supernatant collected after enzymatic treatment. The final $[\text{Ru}(\text{phen})_3]$ in the suspensions after mixing with bacterial suspension was known only for the PS-loaded samples and is given in Table 3.

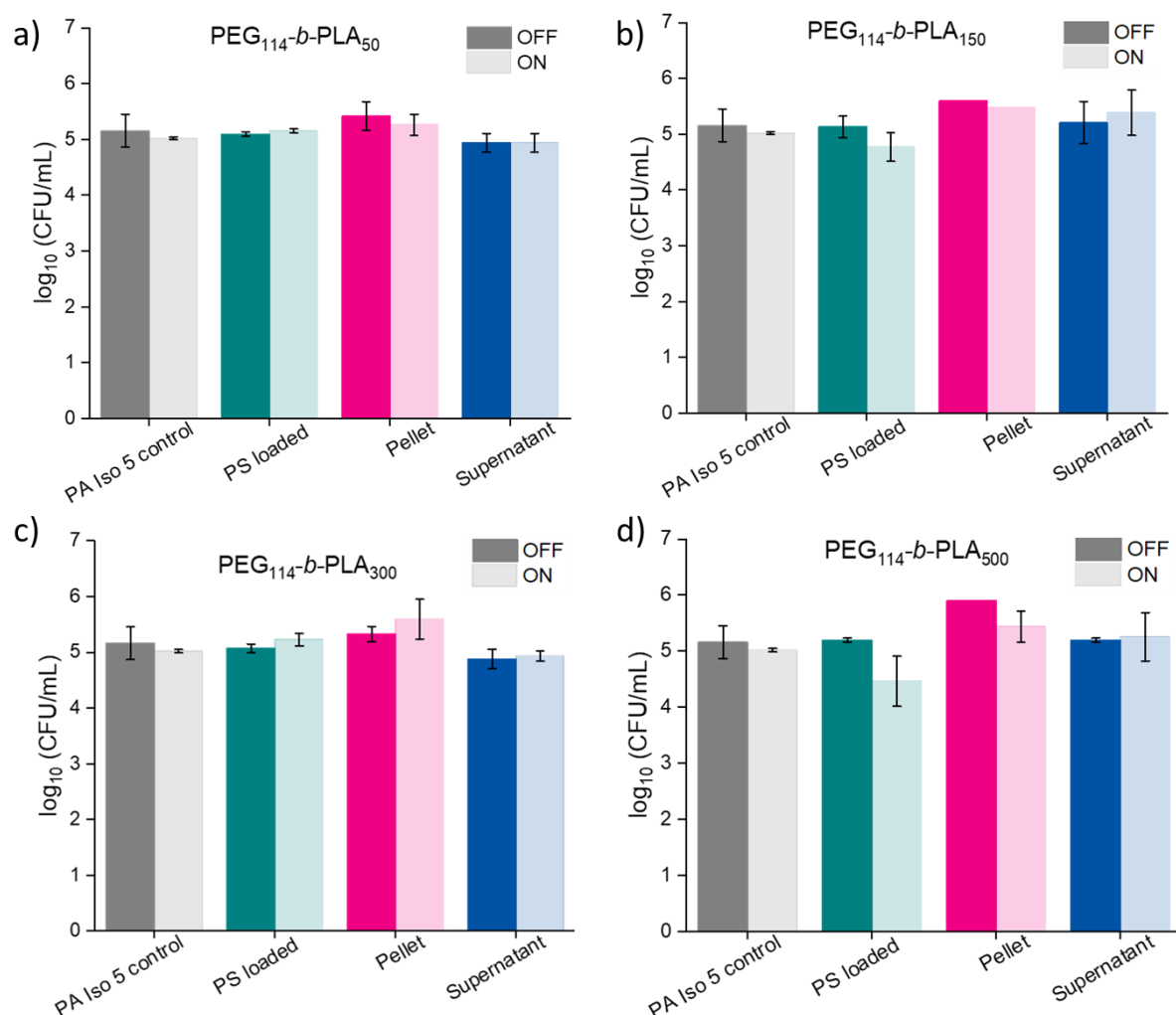


Figure 21: Bar plots of log₁₀ CFU/mL for PS-loaded nanocarriers and PS-loaded nanocarriers after enzymatic treatment (the redispersed pellet after enzymatic treatment ('Pellet') and the collected supernatant ('Supernatant')) treated in the presence of PA Isolate 5 (The final [PS] in PS-loaded carriers after adding bacteria suspension given in Table 5). OFF: non-irradiated samples, ON: samples after 10 min irradiation at $\lambda = 455$ nm.

After 10 min of irradiation, a slight reduction in CFUs was observed for PS-loaded PEG₁₁₄-b-PLA₅₀₀ samples. In contrast, no change in bacterial colony counts was noted between non-irradiated and irradiated samples for other experimental conditions, including the resuspended pellet and the supernatant. Accurately tuning the aPDT parameters for 'pellet' and 'supernatant' samples was challenging because the final PS concentrations in the suspensions after enzymatic treatment could not be precisely determined. Additionally, Ru(phen)₃ is a hydrophilic PS. As indicated by the SOSG probe signals (Figure 13), a hydrophobic PS produced a higher probe signal when released from its loaded nanocarriers following enzymatic treatment. Therefore, investigating the aPDT efficacy through PS release after enzymatic treatment would be more relevant for hydrophobic PS-loaded into nanocarriers.

To enhance the aPDT effects for Ru(phen)₃ loaded nanocarriers, the irradiation time was then increased to 30 min. Figure 22 shows the pictures of the spotting results of bacterial colonies and the corresponding log₁₀ CFU/mL for the samples.

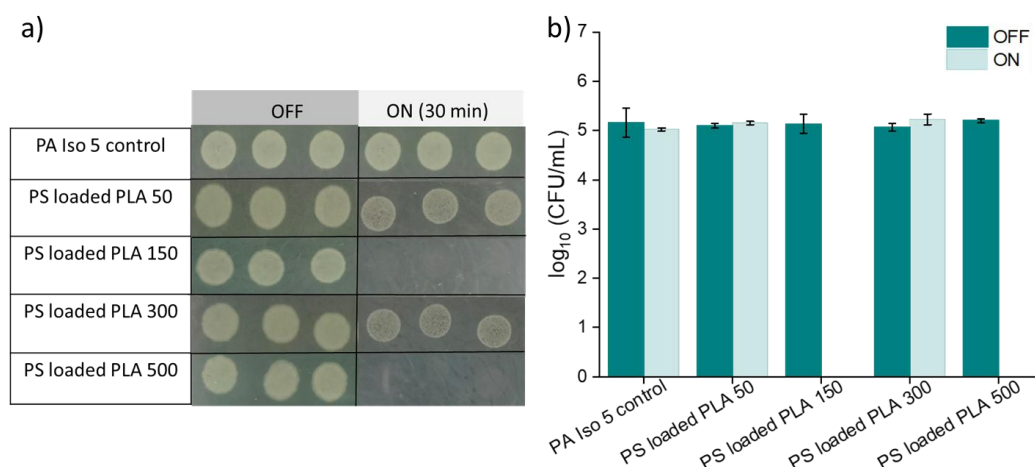


Figure 22: a) Photograph of spotting results, b) log₁₀ CFU/mL bar plots of PS-loaded into assemblies of PEG₁₁₄-*b*-PLA_x (x= 50, 150, 300, and 500) tested against PA Isolate 5. No bar corresponds to no countable colonies after 18 h bacterial colony growth. OFF: non-irradiated samples, ON: samples after 30 min irradiation at $\lambda = 455$ nm.

No bacterial colonies were observed for PS-loaded into PEG₁₁₄-*b*-PLA₁₅₀ micelles and PEG₁₁₄-*b*-PLA₅₀₀ vesicles, when the irradiation time was increased to 30 min. Moreover, the concentration of encapsulated Ru(phen)₃ in suspension of these assemblies was found to be reproducible. Therefore, PS-loaded PEG₁₁₄-*b*-PLA₁₅₀ micelles and PEG₁₁₄-*b*-PLA₅₀₀ vesicles were selected for further aPDT experiments using a 30 min irradiation time.

5.2.5 aPDT effect of PS-loaded micelles and vesicles on bacteria

The PEG₁₁₄-*b*-PLA₁₅₀ micelles and PEG₁₁₄-*b*-PLA₅₀₀ vesicles were loaded with higher concentrations of Ru(phen)₃. The concentration of the Ru(phen)₃ in Ru(phen)₃-loaded micelles was 165.1 ± 15.4 μ M and in Ru(phen)₃-loaded vesicles was 186.9 ± 13.1 μ M. For both the SOSG testing and aPDT experiments, the loaded micelles and vesicles were mixed in 1:1 ratio with the studied different mediums to have their final concentrations to be $\sim$ half of the undiluted loaded micelles and vesicles.

Detection of singlet oxygen

Figure 23 shows the SOSG probe signal of unloaded and Ru(phen)₃-loaded micelles and vesicles incubated with and without bacterial strains PA ATCC and PA Isolate 5 in 0.9% saline. For unloaded micelles (Figure 23a) and vesicles (Figure 23b), a slight increase in probe signal was observed after irradiation. This increase corresponds to the signal originating from the SOSG probe itself, which undergoes photochemistry to a fluorescent product through a different mechanism.²¹ No significant change in fluorescence intensity was observed for the non-irradiated Ru(phen)₃-loaded micelles and vesicles.

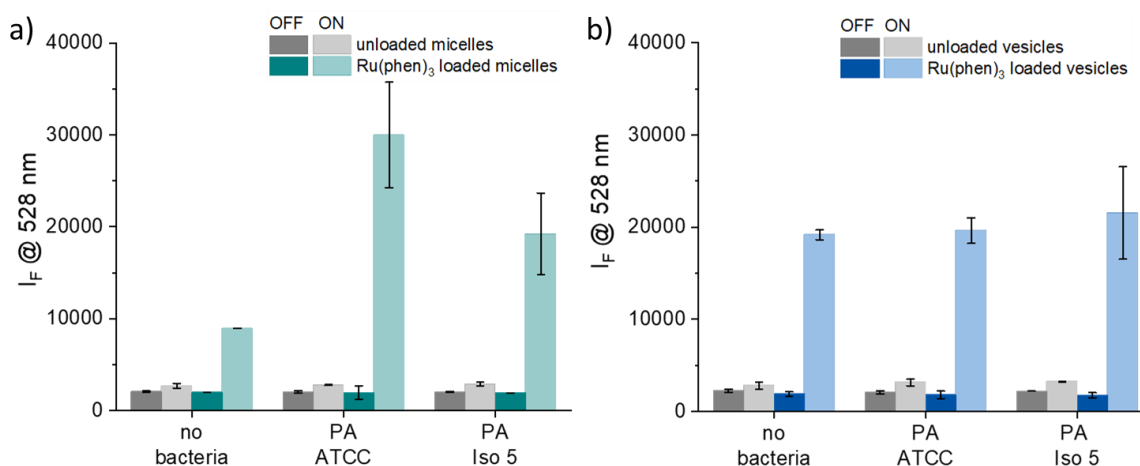


Figure 23: SOSG probe signal for detecting singlet oxygen production by a) unloaded and Ru(phen)₃-loaded micelles in PEG₁₁₄-*b*-PLA₁₅₀ b) unloaded and Ru(phen)₃-loaded vesicles in PEG₁₁₄-*b*-PLA₅₀₀ in the presence of and without PA ATCC and PA Iso 5. (For loaded micelles, final [Ru(phen)₃] in solution = 82.55 ± 7.70 μM; for loaded vesicles, final [Ru(phen)₃] in solution = 93.45 ± 6.55 μM). The incubation time of the samples with the bacteria suspensions was 15 min prior to the addition of the SOSG solution. The samples were irradiated at λ = 455 nm for 30 min (ON) or were incubated in the dark for 30 min (OFF).

In contrast, a pronounced increase in fluorescence intensity was observed for irradiated PS-loaded micelles and vesicles. For loaded micelles, a significantly higher probe signal was observed in samples incubated with bacteria compared to those incubated without bacteria in 0.9% NaCl (Figure 23a). Conversely, for PS-loaded vesicles, no significant difference in probe signal was observed between samples incubated with and without bacterial suspensions (Figure 23b).

The detection of ¹O₂ species depends on various factors, including the concentration of the PS and the local oxygen availability. Additionally, the lifetime of ¹O₂ and its diffusion rate to reach the probe are crucial factors that vary depending on the surrounding media (e.g., viscosity, presence of other reactants, etc.). The fluorescence intensity of the probe also differed between the two bacterial strains. However, no variation in probe signal was observed for loaded vesicles with and without bacteria in sterile water or between the two strains. These findings suggest that the bacterial environment influences micelles differently compared to the polymersomes.

As controls, the probe itself (diluted in tris buffer, pH 7.5) and both bacterial strains (in 0.9% saline) were irradiated in the presence of SOSG (Figure 24a).

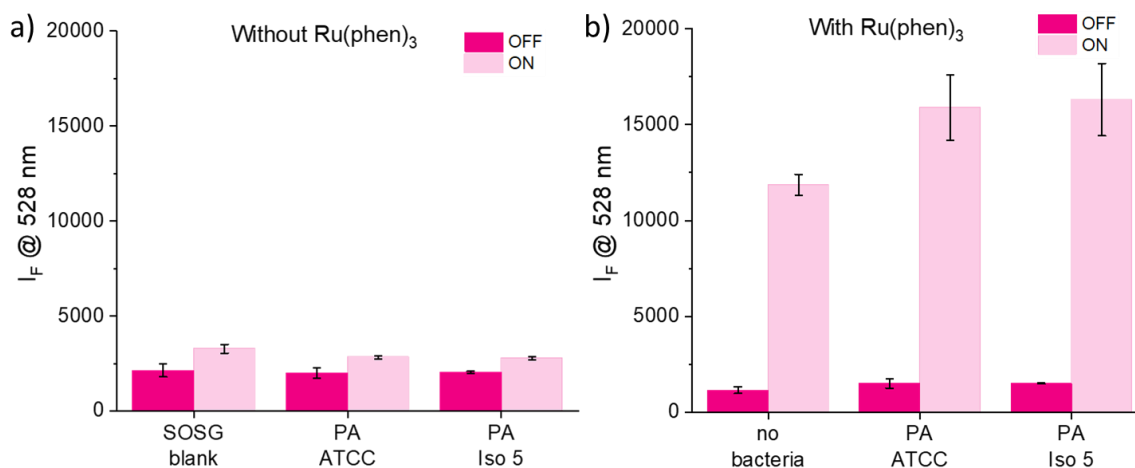


Figure 24: SOSG probe signal for detecting singlet oxygen production by a) SOSG probe and the used bacterial strains PA ATCC and PA Iso 5, b) neat Ru(phen)₃ incubated with and without bacteria strains. The samples were irradiated for 30 min (ON) or incubated in dark for 30 min (OFF).

After irradiation, a slight increase in fluorescence intensity was observed in all cases, corresponding to the previously mentioned side reaction of the SOSG probe during irradiation.¹⁶ In addition, a significant increase in probe signal was observed after irradiation, when neat Ru(phen)₃ was incubated in the absence and presence of the bacterial strains, at concentrations the same to those used in the micelles and vesicles (Figure 25b). A higher probe signal was observed when Ru(phen)₃ was irradiated in bacterial solutions compared to bacteria-free solution. As the intensity was lower in the absence of any polymer, this indicates that the probe may partition into the nanocontainers' PEG corona, which in turn may alter the reacted probes' quantum yield.

aPDT effect against planktonic bacteria

To investigate the aPDT effect of the Ru(phen)₃-loaded micelles and vesicles against PA ATCC and PA Iso 5, the nanocarriers were added to the respective bacteria solutions and were subjected to irradiation following incubation for 15 min. Afterwards, serial dilutions of each sample were performed and three times 5 μ L of each dilution was plated to count the CFU/mL for both the irradiated and non-irradiated samples. For comparison, the aPDT effect of neat Ru(phen)₃ was also investigated. The analysis of the serial dilutions provided the log₁₀ CFU/mL values of the Ru(phen)₃-loaded micelles incubated with each bacteria solution (Figure 25).

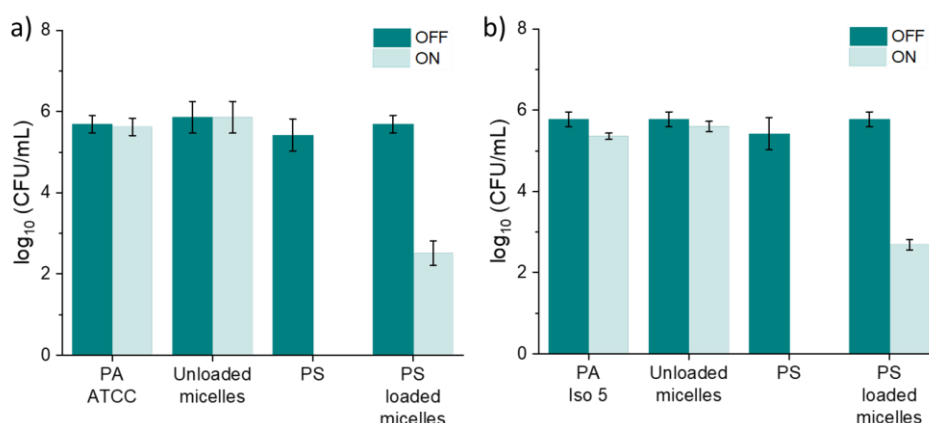


Figure 25: Bar plot of log₁₀ CFU/mL calculated by the plate count method for a. PA ATCC and b. PA Iso 5 incubated with neat Ru(phen)₃ and Ru(phen)₃-loaded micelles for irradiated and non-irradiated samples. (Final neat [Ru(phen)₃] = 82.55 μM, final [Ru(phen)₃] in loaded micelle solution = 82.55 ± 7.70 μM). The samples were irradiated for 30 min (ON) or incubated in the dark for 30 min (OFF). Absence of bar: no countable colonies. The error bars correspond to the standard deviation of technical replicates (*n* = 3).

No effect of irradiation was observed for the bacteria controls and unloaded micelles incubated with bacteria suspension. The non-irradiated samples showed similar CFUs as compared to the corresponding bacteria controls. Neat Ru(phen)₃ showed *no* countable bacteria after the irradiation, while the Ru(phen)₃-loaded micelles showed a > 3 log₁₀ CFU/mL for each bacteria strain after the irradiation.

Similarly, the aPDT effect was investigated for Ru(phen)₃-loaded vesicles. The dilutions were analyzed and the log₁₀ CFU/mL values of the Ru(phen)₃ loaded vesicles were then calculated (Figure 26). No changes in CFU/mL were observed for the non-irradiated samples. Both for neat Ru(phen)₃ and Ru(phen)₃-loaded vesicles, *no* countable bacteria CFUs were observed after the irradiation.

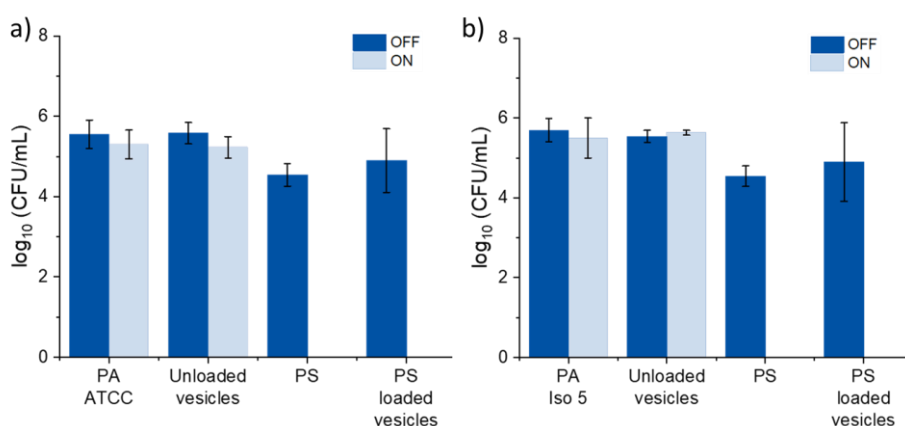


Figure 26: Bar plot of log₁₀ CFU/mL calculated by the plate count method for a. PA ATCC and b. PA Iso 5 incubated with neat Ru(phen)₃ and Ru(phen)₃-loaded vesicles for irradiated and non-irradiated samples. (Final neat [Ru(phen)₃] = 93.45 μM, final [Ru(phen)₃] in loaded vesicle solution = 93.45 ± 6.55 μM). The samples were irradiated for 30 min (ON) or incubated in the dark for 30 min (OFF). Absence of bar: no countable colonies. The error bars correspond to the standard deviation of technical replicates (*n* = 3).

The LRF values were then calculated by comparing each sample with respective non-irradiated bacteria suspension. While calculating LRF values, the LOD values of the biological experiments (67 CFU/mL) were also considered explicitly, which means that the LRF values stated here are lower bounds. Table 4 summarizes the resulting LRFs of Ru(phen)₃ loaded micelles and vesicles.

Table 4: LRF values of the non-irradiated (OFF) and irradiated (ON) Ru(phen)₃-loaded micelles, vesicle, and neat Ru(phen)₃. The LRF was calculated by comparing the data to non-irradiated bacteria controls (n = 3 for loaded micelles and vesicles, n= 6 for neat Ru(phen)₃).

	Ru(phen) ₃ -loaded				Neat Ru(phen) ₃	
	Micelles		Vesicles		OFF	ON
	OFF	ON	OFF	ON		
PA ATCC	-0.1 ± 0.3	3.1 ± 0.2	0.6 ± 0.2	4.7 ± 0.2	2.0 ± 0.2	4.3 ± 0.7
PA Iso 5	0.1 ± 0.2	3.4 ± 0.5	0.8 ± 0.2	4.9 ± 0.2	2.1 ± 0.2	4.4 ± 0.6

For non-irradiated Ru(phen)₃ loaded micelles and vesicles a LRF < 0.8 was observed. For irradiated samples, LRF > 3 was observed for loaded micelles meaning that a bactericidal effect was observed, defined as the inactivation of 99,9 % of the initially viable (here: colony forming) bacteria. For Ru(phen)₃-loaded vesicles, LRF values > 4.7 were observed. The Ru(phen)₃-loaded vesicle formulations thus showed much higher bacteria inactivation capacity as compared to Ru(phen)₃-loaded micelles and even to neat Ru(phen)₃. The mechanisms for this improvement via the nanocarrier require further investigation. One may hypothesize that, apart from stabilizing the PS itself, the nanocarriers may affect the interaction of Ru(phen)₃ with the bacterial cell wall, which depends both on the bacterial pathogen and its wall structure (Gram-positive or Gram-negative) and the surrounding media.^{29,30}

Comparing the SOSG probe signal intensities of PS-loaded micelles and vesicles incubated with bacteria solutions, higher SOSG signal intensities were observed for loaded micelles as compared to loaded vesicles after irradiation, while the aPDT results show the opposite trend. Higher LRFs were observed for loaded vesicles as compared to micelles. Therefore, it is not *a priori* valid to directly correlate the SOSG probe signal with the aPDT efficiency of the systems. Several other factors are required to be considered, such as e.g. the interaction of bacteria with the nanocarriers, the availability of the produced singlet oxygen species to reach the bacteria,³¹ and various effects that reduce the quantum yields of the reporter probe.

As a final goal with high medical impact aPDT should achieve a complete eradication of bacteria in order to avoid a recolonisation of infected tissue, wound dressings etc. Although also PS-loaded micelles resulted in a clear bactericidal effect with a LRF >3, only the Ru(phen)₃-loaded vesicle formulations afforded a complete killing. This effect even exceeded

the photodynamic inactivation (PDI) efficiency of neat PS (Table 4). Hence encapsulating PS in vesicles may enhance the PDI efficiency for planktonic *Pseudomonas aeruginosa*. A similar effect was observed by Mirzahosseini et al., who compared the aPDT effect of curcumin and curcumin encapsulated in silica nanoparticles.³² They observed a further reduction in the CFU/mL of planktonic bacteria for curcumin encapsulated silica nanoparticles as compared to neat curcumin. The curcumin silica nanoparticles were shown to improve the activity of curcumin due to good uptake and controlled release of curcumin.

The Ru(phen)₃-loaded micelles showed similar aPDT efficiency of 99.9% inactivation (3-log reduction) compared to the comparable system of lipase-sensitive PEG-*b*-PCL micelles reported by Guo et al.³³ However, the Ru(phen)₃-loaded PEG₁₁₄-*b*-PLA₅₀₀ polymersomes showed an antimicrobial effect with a > 4.7-log reduction, which is comparable, but requires less light intensity compared to reports for similar polymeric nanocarrier systems. Microparticles irradiated with red light power 9.28 mW/cm² for 30 min or other types of inorganic nanoparticles e.g. gallium-substituted silver nanoparticles irradiated with nearly 50 times higher light intensity (140 mW/cm²), but only 10s irradiation of blue light like used in this study, were reported before.^{34,35}

The herewith reported systems could be further improved by the use of more efficient ruthenium-based photosensitizers as reported in recent literature^{20,31,36,37} or photosensitizers, which have been shown to be efficient against various bacteria e.g. methylene blue or rose bengal. Also PS molecules, which are excited at longer wavelengths, could be used for encapsulation, since those PS might be easier to excite within biological media or tissues due to the more pronounced penetration depths of light.

In summary, the antimicrobial activity observed herein can be explained by the underlying photodynamic mechanism involving photosensitizer localization, reactive oxygen species (ROS) generation, and bacterial damage. Upon blue light irradiation ($\lambda = 455$ nm), the Ru-based PS's are photoexcited, transitioning from the ground state to an excited singlet state, followed by intersystem crossing to a long-lived triplet state. This triplet state facilitates energy transfer to molecular oxygen, producing singlet oxygen (¹O₂), the primary cytotoxic ROS responsible for bacterial killing in aPDT. Given the short lifetime (~3-4 μ s) and limited diffusion radius (~10–20 nm) of ¹O₂ in aqueous media, the spatial proximity between the PS and the bacterial cell surface is critical.

Furthermore, while enzymatic degradation of the PLA segments was hypothesized to trigger enhanced PS release and thereby improve aPDT efficacy, no substantial improvement was observed, suggesting that in these systems, PS is already sufficiently available for ROS generation.

The PS-loaded nanocarriers may interact with the bacterial membrane, allowing locally generated $^1\text{O}_2$ to cause oxidative damage to vital cellular components, including membrane lipids, proteins, and DNA. In micelles, PS molecules are more surface-accessible, leading to measurable $^1\text{O}_2$ fluorescence signals upon irradiation. In contrast, vesicles may encapsulate PS in a more protected environment, but their larger structure and possible closer association with bacterial membranes can enhance contact-mediated ROS delivery, explaining their superior bacterial killing despite lower fluorescence readouts (Figure 23).

The higher log reduction factor (LRF) achieved by vesicle-based delivery compared to micelles or free PS highlights the synergistic role of the nanocarrier architecture in enhancing PS stability, local concentration at the bacterial surface, and possibly PS-bacteria interactions, leading to efficient photodynamic inactivation.

5.3 Conclusion

The different Ru-based PS were evaluated for their singlet oxygen production and aPDT efficacy. The photodynamic eradication of *Pseudomonas aeruginosa* lab strain (PA ATCC 19660) and a clinically relevant CF-Isolate (CH2678) was achieved *in vitro* using blue light irradiation. Investigating irradiation parameters and selecting suitable PEG-*b*-PLA nanocarrier architectures were essential for maximizing aPDT outcomes. Notably, PS-loaded vesicles showed a greater photodynamic inactivation compared to PS-loaded micelles, achieving a > 4.7 log reduction in viable bacterial counts for both strains. This enhanced efficacy is attributed to the higher PS loading capacity within the vesicular structures. The findings underscore the potential of Ru-based PS encapsulated within polymeric nanocarriers for targeted and effective bacterial eradication. The study provides valuable insights into the design and application of advanced nanocarrier systems to enhance aPDT outcomes, paving the way for future therapeutic developments in aPDT.

5.4 Materials and methods

5.4.1 Materials

Samples

Tris(1,10-phenanthroline) ruthenium (II) bis(hexafluorophosphate) ($\text{Ru}(\text{phen})_3$, >98%) was purchased from TCI chemicals (Germany). Singlet Oxygen Sensor Green SOSG (Thermo Fischer Scientific, Eugene, OR, USA), Methanol (>99.8%, VWR, Germany), Tris buffer 100 mM pH 7.4 (Millipore, MA, USA), dimethyl sulfoxide (DMSO, 99% Sigma Aldrich, Germany), Lysogeny broth (LB, Luria/Miller: 10g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7.0 $\pm$ 0.2, Carl Roth GmbH, Germany), LB agar (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl,

aPDT effect of encapsulated Ru-based photosensitizers against *Pseudomonas aeruginosa*

15 g/L agar-agar, pH 7.0 ± 0.2 Carl Roth, Germany), sterile water and 0.9% saline (Versylene Fresenius, Germany), transparent flat-bottom 96 well plate (Sarstedt), and cell culture 96 black half area μ -clear well plate (Greiner Bio-One, Germany) were purchased from mentioned suppliers. Milli-Q water (for preparing LB medium and LB agar plates) was drawn from a Millipore Direct Q8 system with a resistivity of 18.2 M Ω cm (Millipore advantage A10 system, Schwalbach, with Millimark Express 40 filter, Merck, Germany).

Bacterial strains

Pseudomonas aeruginosa lab strain ATCC 19660 (isolated from sepsis in Lima, Peru, and provided by ATCC) here noted as PA ATCC and *Pseudomonas aeruginosa* cystic fibrosis patient strain CH2678 (isolated from sputum, isolated at Charité Berlin, provided by Prof. Susanne Häußler, Helmholtz Centre for Infection Research, Braunschweig, Germany), denoted here as PA Iso 5, were used in this study.

Sample preparation

Synthesis of Ru-based photosensitizer solutions

The Ru-based photosensitizers were synthesized by Prof. Dr. Gilles Lemerrier (Université de Reims Champagne-Ardenne, France). 5 mM stock solutions of Ru-based photosensitizers were prepared in DMSO. For singlet oxygen detection and absorbance measurements, the desired final solution concentrations were prepared in tris buffer (pH 7.4) except for MF38 (prepared in THF).

5.4.2 Methods

UV-Vis spectroscopy

A Varian Cary 50 Bio spectrometer (Mulgrave, Victoria, Australia) was used for the absorbance measurements. The spectrum was recorded using a 1 cm path-length quartz cuvette at 25 °C with a scan rate of 600 nm/min.

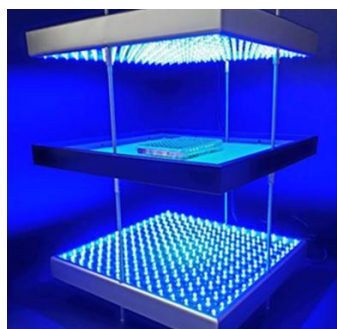
Fluorescence spectroscopy

The fluorescence intensity was measured using a microplate reader (Tecan SAFIRE, Tecan, Switzerland). The measurements were recorded using cell culture 96 black half area μ -clear well plates with fluorescence top reading mode at $\lambda_{\text{ex}} = 504$ nm and $\lambda_{\text{em}} = 528$ nm.

Irradiation experiments

A homemade LED irradiation setup was used to perform the irradiation experiments. The setup includes two panels of 225 blue LEDs (14 W, $\lambda = 450$ -460 nm, HQR LED plant grow panel lamp system, US) facing each other at adjustable distances as shown in Figure The panels were placed in such a way on top and bottom to have an exposure area in the center to achieve irradiation from both sides of the well plate. The irradiation intensity at the exposure

area was adjusted to 2.7- 3.0 mW/cm², as measured using a PM 160 wireless handled power meter (Thorlabs, Bergkirchen, Germany).



$\lambda = 455 \pm 5 \text{ nm}$

Figure 27: The LED set-up used for irradiation.

For irradiation, the sample well plate was placed in the exposure area and was irradiated at room temperature for different irradiation times. To assess the effect of irradiation, treated samples were compared with samples not exposed to light. In the chapter, these conditions are referred to as 'OFF' (non-irradiated samples) and 'ON' (irradiated samples).

Detection of singlet oxygen production

To detect the production of singlet oxygen, the fluorescent singlet oxygen sensor green SOSG probe was used. In all experiments, 90 μL of the PS samples were mixed with 10 μL of 100 μM SOSG probe (final SOSG concentration: 10 μM). The samples were incubated in the dark for 15 min and then irradiation was performed. The SOSG fluorescence signal for irradiated and non-irradiated samples was measured at an excitation of 504 nm and emission at 528 nm. For both the SOSG testing and aPDT experiments, the loaded micelles and vesicles were mixed in 1:1 ratio with the studied different mediums to have their final concentrations to be $\sim$ half of the undiluted loaded micelles and vesicles.

aPDT effect on planktonic bacteria

Overnight cultures were prepared by taking single colonies of each bacterial strain in 5.0 mL LB followed by incubating at 37°C for 18 h while shaking at 200 rpm. Then 1.0 mL of the cultures were centrifuged at 10,000 rpm for 5 min. The supernatant was discarded and the pellet was resuspended in 0.9% NaCl. The OD at 600 nm of each bacteria solution was adjusted to 0.6, corresponding to $\sim 10^8$ CFU/mL. The 100x dilution was made in 0.9% NaCl to obtain a final working solution concentration of both bacterial strains of $3 \times 10^6 \pm 2 \times 10^6$ CFU/mL. Afterward, 25 μL of both bacteria solutions were distributed in a 96 well-plate containing 25 μL of PS solutions. Each sample condition was performed in technical triplicate (3 wells), separately for irradiated and non-irradiated samples. The samples were incubated for 15 min at room temperature and then the irradiation was performed. To evaluate the aPDT effect, 5 μL of each technical replicate (well) of the irradiated and non-irradiated undiluted and

serially diluted samples (up to 10⁵ fold) were spotted three times on agar plates (15 µL in total) that results in a detection limit of the method of 67 CFU/mL. The plates were incubated overnight at 37°C and the next day bacteria colonies were counted for CFU/mL calculations of all sample conditions. The log₁₀ CFU/mL and the LRF were then calculated. For the LRF the respective non-irradiated bacteria suspension was taken as the control. The arithmetic mean and the standard deviation were calculated from 3 times independently performed experiments, with each experiment based on 3 technical replicates.

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6 aPDT with Ru(phen)₃-loaded vesicles in artificial sputum medium and within biofilms

This Chapter presents an investigation of the antimicrobial photodynamic therapy (aPDT) parameters of Ru(phen)₃ and Ru(phen)₃-loaded vesicles against planktonic *Pseudomonas aeruginosa* (*P. aeruginosa*) and against *P. aeruginosa* biofilms. For experiments with planktonic bacteria, *P. aeruginosa* suspensions were prepared in artificial sputum medium (ASM) to replicate the conditions of a Cystic Fibrosis (CF) lung environment. Additionally, the effect of pre-incubation time of Ru(phen)₃ with biofilms on the efficacy of aPDT was studied.

6.1 Introduction and objectives

P. aeruginosa is a Gram-negative opportunistic pathogen commonly associated with chronic respiratory infections, particularly in individuals with CF.¹ The CF lung environment is characterized by thick, viscous sputum that serves as a nutrient-rich medium for bacterial colonization and biofilm formation.¹ To study the behavior and physiology of *P. aeruginosa* under conditions that closely mimic the CF lung, researchers have developed artificial ASM.^{2,3} ASM contains mucin, DNA, amino acids and lipids.³ These *in-vitro* models aim to replicate the complex chemical and physical properties of CF sputum, providing a platform for investigating bacterial adaptation, virulence, and responses to therapeutic interventions.^{4,5,6}

Moreover, *P. aeruginosa* poses a significant threat in clinical settings due to its ability to form biofilms. Biofilms are structured communities of bacteria encased in a self-produced extracellular polymeric substance (EPS), which provides resistance to antibiotics and host immune responses.^{7,2} State of the art *P. aeruginosa* biofilms are commonly associated with chronic infections, such as those seen in cystic fibrosis patients, burn wounds, and medical device-related infections.^{8,9}

The eradication of chronic *P. aeruginosa* infections and biofilms remains a major challenge due to their intrinsic resistance mechanism. Conventional antibiotic therapy often fails, as biofilm-embedded bacteria exhibit up to 1,000 times greater resistance than their planktonic counterparts.¹⁰ Several factors contribute to the resistance to antibiotic treatment. It includes overproduction of exopolysaccharide alginate, which reduces penetration of many antibiotics, in particular the aminoglycosides,¹¹ and reduced metabolic activity.¹² Several strategies have been explored to address this issue, including antimicrobial peptides,¹³ bacteriophage therapy,¹⁴ and quorum sensing inhibitors.¹⁵ Furthermore, CF patients receive frequent courses of antibiotics which result in strong selective pressure for the emergence of isolates with reduced susceptibility to many antibiotics.^{16,17,18,19}

As discussed in Chapter 2 and 5, aPDT has emerged as a potential alternative to traditional methods for treating bacterial infections, including those caused by *P. aeruginosa*. aPDT involves the administration of photosensitizers which, upon light activation, generate reactive oxygen species that disrupt bacterial membranes and biofilms. The effectiveness of aPDT depends on the type of photosensitizer used, the wavelength of light, and the oxygen availability within the biofilm and the ASM.^{20,21}

Several studies have utilized ASM to evaluate strategies aimed at eradicating *P. aeruginosa*. Donnelly et al. assessed the diffusion of two photosensitizers-toluidine blue O and meso-tetra (N-methyl-4-pyridyl) porphine tetra tosylate - through artificial CF mucus.²² Upon activation with red light (635 nm), they achieved significant bacterial kill rates (>99%) in both planktonic and biofilm cultures of *P. aeruginosa*. However, higher concentrations of photosensitizers were required to achieve reasonably high kill rates (>80%) in the presence of artificial mucus. Many known photosensitizers used for aPDT show only limited biocompatibility, limited solubility in aqueous or biological media, relevant dark toxicity and reduced photoactivity in the aggregated state, which leads to difficulties in applications.^{23,24}

Among synthetic photosensitizers, metal complexes such as ruthenium (II) complexes (Ru(II)) have been extensively studied for PDT effects against fatal cancers, and some lead complexes undergo phase II clinical studies for PDT treatment of bladder cancer.^{25,26,27} Recently, Youf et al. used Ru(II) based photosensitizers (including commercially available Ru(phen)₃) to study aPDT efficacy in the presence of ASM and within biofilms in prospects for application in CF lung infections.^{28,29} In a similar direction to achieve optimal performance of Ru(II) based PS in medical applications, as reported in Chapter 5 investigated the encapsulation of commercially available Ru(phen)₃ in polymeric assemblies and reported optimal aPDT conditions for efficient photodynamic inactivation of *P. aeruginosa* CF isolate in its planktonic form.³⁰ Taking this initial results a step further, in the investigations reported in this Chapter, I focused on studying the aPDT effects of Ru(phen)₃ loaded vesicles (see Chapter 5) against *P. aeruginosa* CF isolate prepared in an artificial sputum medium (ASM) and against *P. aeruginosa* biofilms.

6.2 Results and discussion

The impact of the various parameters for aPDT on its efficacy were investigated using neat Ru(phen)₃ and comparing this to the efficacy of Ru(phen)₃-loaded PEG₁₁₄-*b*-PLA₅₀₀ vesicles. In particular the efficacy against *P. aeruginosa* CF isolates prepared in ASM and against biofilms were studied. The Ru(phen)₃-loaded PEG₁₁₄-*b*-PLA₅₀₀ vesicles used in this Chapter were provided by Max Müller.

6.2.1 Investigation of aPDT parameters in the presence of ASM

The work discussed in Chapter 5 showed the aPDT efficacy of Ru(phen)₃-loaded PEG₁₁₄-*b*-PLA₅₀₀ vesicles against planktonic bacteria in 0.9% saline. For more realistic tests, ASM was used here. Hence different batches of ASM were prepared to assess batch-to-batch variability in aPDT efficacy and to check the stability of ASM over time. Firstly, the aPDT efficacy of neat Ru(phen)₃ was tested at different concentrations and irradiation times. Then the aPDT effects of neat Ru(phen)₃ were compared with Ru(phen)₃-loaded vesicles.

aPDT of neat Ru(phen)₃ in artificial sputum medium batch 1

Bacterial suspensions were prepared by centrifuging an overnight culture of PA Iso 5 to obtain pellets, which were then resuspended in ASM. Neat Ru(phen)₃ in water was mixed with the bacterial suspension in 1:1 ratio, resulting in final concentrations to be ~ half of the undiluted Ru(phen)₃. Ru(phen)₃ was incubated with bacteria suspension for 15 min (final concentrations of Ru(phen)₃: 50, 100, and 200 μ M) followed by irradiation for different times. To assess bacterial viability, serial dilutions of the treated samples were performed, and 10 μ L aliquots of each dilution were plated onto LB agar for non-irradiated and irradiated samples. The corresponding log₁₀ CFU/mL values are given in Figure 1.

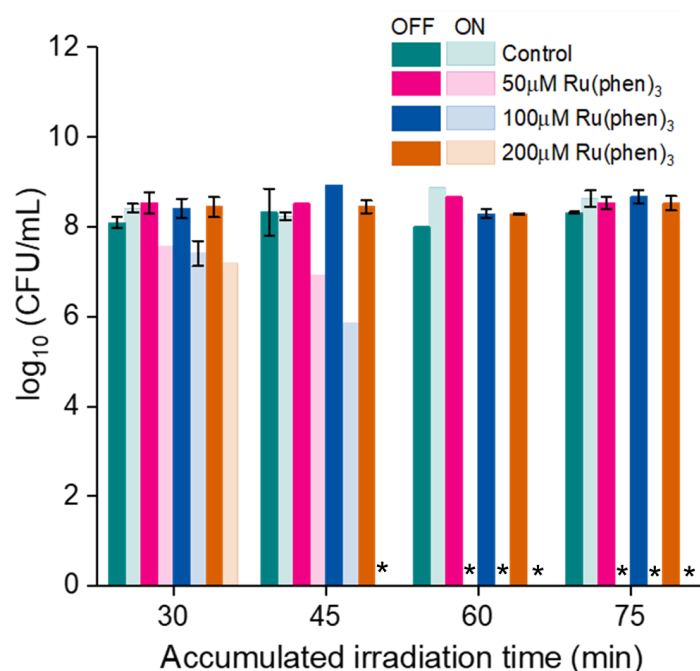


Figure 1: Bar plots of log₁₀ CFU/mL for different Ru(phen)₃ concentrations tested against PA Iso 5 prepared in ASM. The samples were either incubated in the dark (OFF) or irradiated for different irradiation times (ON) at $\lambda = 455$ nm. The error bars correspond to the standard deviation of technical replicates ($n = 3$). No bar corresponds to no countable colonies after 18 h bacterial colony growth (marked as *).

No effect of irradiation on bacteria count was observed in the bacterial controls at different irradiation times. The non-irradiated samples containing Ru(phen)₃ showed similar CFU counts comparable to those of corresponding bacteria controls. After 30 min irradiation, a

slight reduction in CFUs was observed for all Ru(phen)₃ concentrations, suggesting that either the generated singlet oxygen species were insufficient to cause lethal damage within this timeframe, or that the bacteria exhibited partial tolerance mechanisms.

When the irradiation time was increased to 45 min, complete bacterial eradication was observed at 200 μ M Ru(phen)₃, indicating that higher Ru(phen)₃ concentrations enhance singlet oxygen production, thereby increasing oxidative stress on bacterial cells. This leads to more effective cell membrane damage, protein oxidation, and DNA disruption.^{31,32,33} For 100 and 50 μ M Ru(phen)₃, a slight improvement in aPDT efficacy was observed after 45 min irradiation. However, after 60 min, no viable colonies were observed at either concentration. Improving aPDT efficacy with increasing irradiation time and Ru(phen)₃ concentration suggests that a critical oxidative stress level is crucial to ensure effective bacterial eradication.

The composition of ASM influences aPDT efficacy as it provides a nutrient-rich environment containing DNA, mucin, and egg yolk emulsion, which can scavenge singlet oxygen species or hinder the diffusion of both photosensitizer and singlet oxygen.²² Additionally, diethylenetriaminepentaacetic acid (DTPA) in ASM chelates metal ions, creating a controlled environment for *in-vitro* bacterial studies. This chelation helps regulate the availability of essential nutrients, such as iron. Neve et al. reported differences in *P. aeruginosa* secondary metabolite production in response to different iron concentrations in ASM, highlighting the importance of maintaining controlled metal ion availability which significantly influence bacterial growth and the production of virulence factors.³ Since Ru(phen)₃ is a metal-based complex, DTPA may also chelate with it, reducing the free photosensitizer concentration and consequently diminishing its aPDT efficacy in ASM.

To evaluate ASM stability, aPDT experiments were done with one-week-old ASM. PA ATCC prepared in ASM was treated with 50 μ M Ru(phen)₃ and incubated for 15 min incubation followed by irradiation at different times. As a control, PA ATCC prepared in 0.9% saline was similarly treated with 50 μ M Ru(phen)₃. The corresponding log₁₀ CFU/mL values for both non-irradiated and irradiated samples are shown in Figure 2.

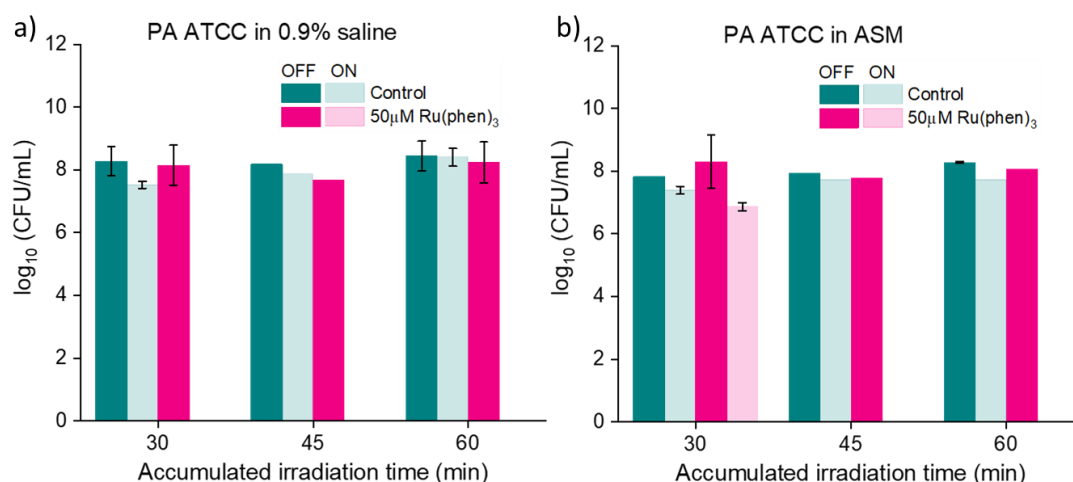


Figure 2: Bar plots of log₁₀ CFU/mL for 50 μM Ru(phen)₃ tested against PA ATCC prepared in a) 0.9% saline and b) one-week-old ASM. The samples were either incubated in the dark (OFF) or irradiated for different irradiation times (ON) at $\lambda = 455$ nm. The error bars correspond to the standard deviation of technical replicates ($n = 3$). No bar corresponds to no countable colonies after 18 h bacterial colony growth.

For both bacterial controls in saline and ASM, no aPDT effect was observed after different irradiation times. Similarly, non-irradiated samples containing Ru(phen)₃ showed CFUs compared to the corresponding bacteria controls. In saline, complete bacterial eradication was achieved after 30 min irradiation (Figure 2a), mainly due to the absence of interfering components in the media, which allowed efficient singlet oxygen generation and enhanced the bacterial eradication. In ASM, complete killing required 45 min irradiation. These findings highlight the significant influence of the suspension medium on aPDT efficacy. Previous studies reported on the diffusion of Ru(phen)₃ through the ASM layer, and observed that it diffuses only 11% through a 1 mm layer of ASM, compared to 59% in the absence of ASM²⁹, which further limits its availability for photodynamic activity.

A comparison of Figure 2b with aPDT results from freshly prepared ASM with 50 μM Ru(phen)₃ (Figure 1) revealed that complete bacterial eradication required 60 min of irradiation in fresh ASM, whereas in one week-old ASM, complete killing was observed after 45 min. This suggests that freshly prepared ASM retains intact organic components capable of scavenging singlet oxygen and interacting with photosensitizer, thereby reducing aPDT efficacy and necessitating prolonged irradiation. Over time, ASM components undergo degradation, decreasing their singlet oxygen quenching capacity and improving photosensitizer availability, leading to more effective bacterial inactivation with shorter irradiation times. While direct studies on ASM degradation are limited, similar reports on similar biological sputum indicate mucin degradation, which may also occur in ASM,^{34,35} altering its physical and chemical properties and subsequently influencing aPDT outcomes.

aPDT of neat Ru(phen)₃ in artificial sputum medium batch 2

For further investigation, a fresh batch of ASM was prepared, and the effects of different Ru(phen)₃ concentrations were tested against PA ATCC and PA Iso 5. The bacterial suspensions were incubated with Ru(phen)₃ at final concentrations of 25, 50, and 100 μ M for 15 min, followed by irradiation at different times. Serial dilutions of the treated samples were performed and 10 μ L aliquots of each dilution were plated onto LB agar for both non-irradiated and irradiated samples. The corresponding log₁₀ CFU/mL values are given in Figure 3.

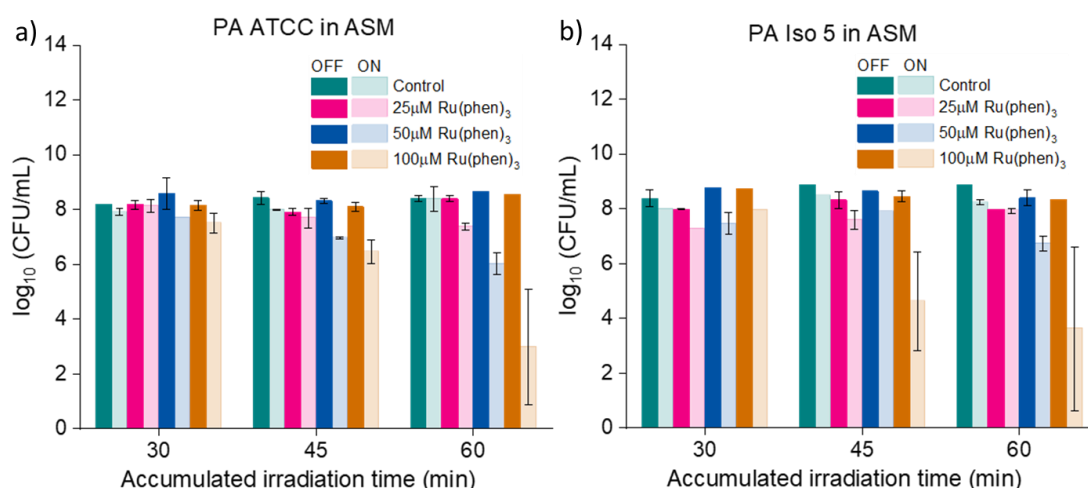


Figure 3: Bar plots of log₁₀ CFU/mL for different Ru(phen)₃ concentrations tested against a) PA ATCC and b) PA Iso 5 prepared in ASM. The samples were either incubated in the dark (OFF) or irradiated for different irradiation times (ON) at $\lambda = 455$ nm. The error bars correspond to the standard deviation of technical replicates ($n = 3$).

For both bacterial controls, no effect of irradiation was observed for different irradiation times. After 30 min irradiation, a slight reduction in CFUs was observed for all Ru(phen)₃ concentrations. Increasing both irradiation time and Ru(phen)₃ concentration enhanced the aPDT effect, however complete bacterial eradication was not achieved even after 60 min irradiation. Notably, at 100 μ M Ru(phen)₃, substantial variability was observed, as indicated by large error bars, suggesting inconsistencies in bacterial colony counts within the replicates.

Comparison of aPDT of neat Ru(phen)₃ with ASM batch 1 and batch 2

The aPDT efficacy of freshly prepared ASM batch 1 and ASM batch 2 was compared for PA Iso 5. Figure 4 shows the log₁₀ CFU/mL values obtained for Ru(phen)₃ (final concentrations of 50 and 100 μ M) following irradiation at different time intervals.

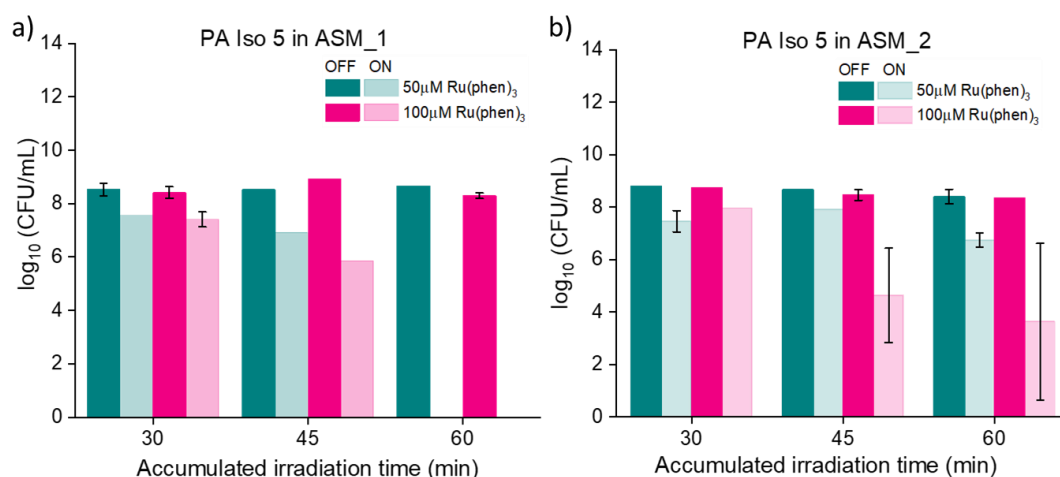


Figure 4: Bar plots of log₁₀ CFU/mL for Ru(phen)₃ (final concentrations 50 and 100 μM) tested against PA Iso 5 prepared with a) ASM batch 1 and b) ASM batch 2. The samples were either incubated in the dark (OFF) or irradiated for different irradiation times (ON) at $\lambda = 455$ nm. The error bars correspond to the standard deviation of technical replicates ($n = 3$). No bar corresponds to no countable colonies after 18 h bacterial colony growth.

For ASM batch 1, no countable colonies were detected after 60 min irradiation with both Ru(phen)₃ concentrations. In contrast, for ASM batch 2, bacterial colonies were observed even after prolonged irradiation (60 min) indicating batch-to-batch variability in aPDT efficacy. These differences highlight the necessity of using a single freshly prepared ASM to ensure consistency in experimental outcomes.

Until now, the aPDT experiments have been performed using neat Ru(phen)₃. Next the impact of encapsulation on the aPDT efficacy was studied.

aPDT of neat Ru(phen)₃ vs Ru(phen)₃-loaded vesicles in artificial sputum medium batch 3

A fresh batch of ASM was prepared to evaluate the aPDT efficacy of Ru(phen)₃-loaded vesicles against PA ATCC and PA Iso 5 in both ASM and 0.9% saline. The detection of generated singlet oxygen was also done using Singlet Oxygen Sensor Green (SOSG) probe. For both the SOSG testing and aPDT experiments, the vesicles were mixed in a 1:1 ratio with the respective media, resulting in final concentrations to be ~ half of the undiluted loaded vesicles.

Detection of singlet oxygen

The neat Ru(phen)₃ and Ru(phen)₃-loaded vesicles were incubated with bacterial suspensions for 15 min (final concentrations in solution: 132 μM). The singlet oxygen production was detected by adding SOSG probe solution (final SOSG concentration: 10 μM) to the samples, followed by irradiation. Figure 5 shows the fluorescence response of the SOSG probe for bacterial suspensions in ASM and saline over different irradiation time intervals.

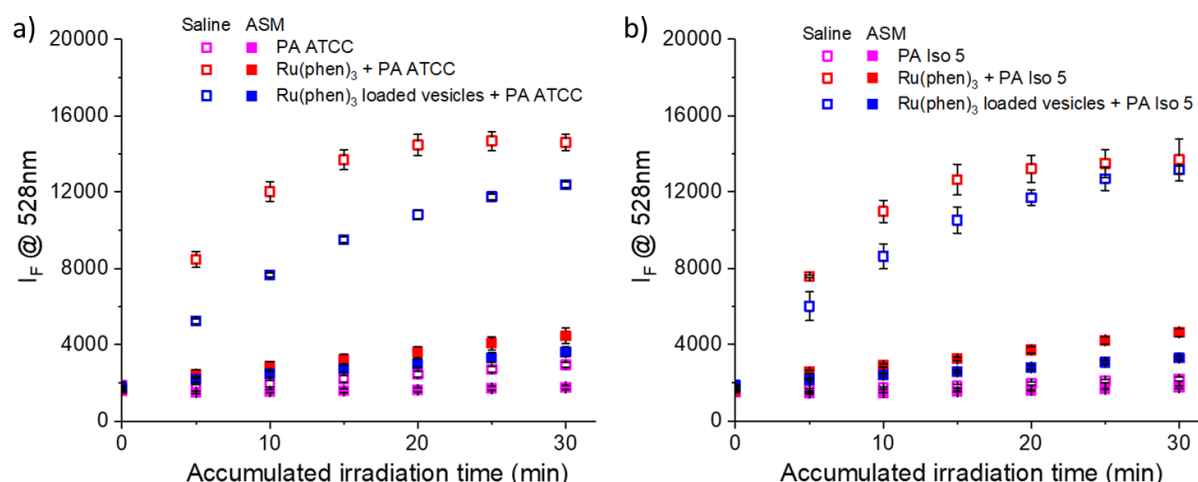


Figure 5: SOSG probe signal for detecting singlet oxygen production by Ru(phen)₃-loaded vesicles and neat Ru(phen)₃ tested against a) PA ATCC in the presence of ASM and saline and b) PA Iso 5 in the presence of ASM and saline. The samples were irradiated for different time intervals at $\lambda = 455$ nm.

For both bacterial suspensions in ASM and saline, no increase in signal intensity was observed in the absence of Ru(phen)₃. In saline, the presence of Ru(phen)₃ led to a pronounced increase in fluorescence intensity over time, with higher signal response in the case of neat Ru(phen)₃ compared to Ru(phen)₃-loaded vesicles. However, when Ru(phen)₃ was present in bacterial suspensions in ASM, only a slight increase in the probe signal was observed for the studied irradiation times. This reduced fluorescence response is likely due to the complex composition of ASM, which may lead to singlet oxygen quenching, restricted diffusion, or reduced availability of free photosensitizer within the medium. Consequently, aPDT efficacy in ASM is expected to be lower than in saline.

aPDT effect of neat Ru(phen)₃ vs Ru(phen)₃-loaded vesicles against planktonic bacteria in ASM

To assess aPDT effect, Ru(phen)₃-loaded vesicles and neat Ru(phen)₃ were incubated with bacterial suspensions in ASM for 15 min (final Ru(phen)₃ concentration in solution: 132 μ M) followed by irradiation at different times. As a control, aPDT experiments were also done with bacterial suspensions in 0.9% saline. The serial dilutions of the treated samples were done and 10 μ L aliquots of each dilution were plated onto LB agar for non-irradiated and irradiated samples. The resulting log₁₀ CFU/mL values for PA ATCC in saline and ASM are given in Figure 6.

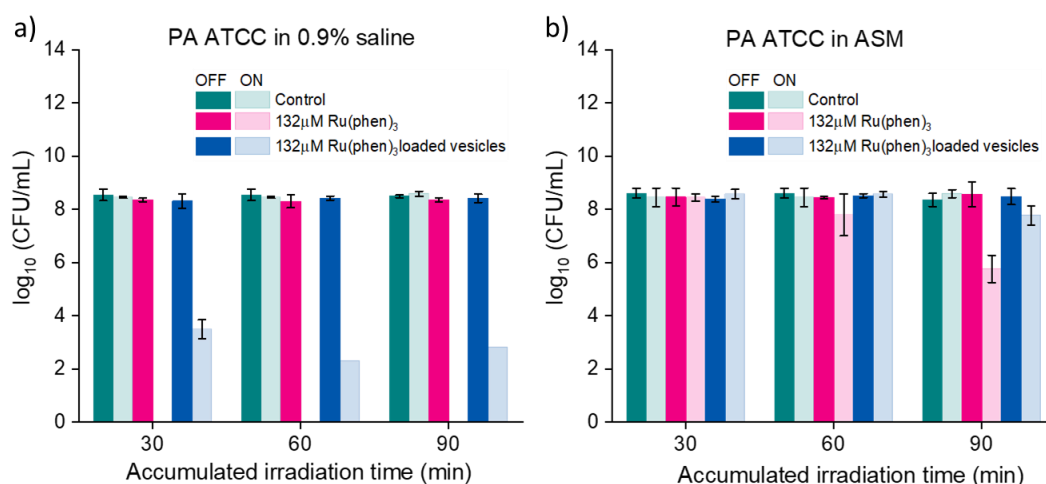


Figure 6: Bar plots of log₁₀ CFU/mL for Ru(phen)₃-loaded vesicles and neat Ru(phen)₃ tested against PA ATCC prepared with a) 0.9% saline and b) ASM. The samples were either incubated in the dark (OFF) or irradiated for different irradiation times (ON) at $\lambda = 455$ nm. The error bars correspond to the standard deviation of technical replicates ($n = 3$). No bar corresponds to no countable colonies after 18 h bacterial colony growth.

For PA ATCC prepared in saline (Figure 6a), no viable colonies were observed after 30 min irradiation with neat Ru(phen)₃, corresponding to >7 LRF (obtained after considering the detection limit of colony counting under the experimental conditions). In case of Ru(phen)₃ loaded vesicles, >5 LRF was observed after 30 min, which increased to >6 after 60 and 90 min irradiation. In contrast, for PA ATCC in ASM (Figure 6b), no aPDT effect was observed with Ru(phen)₃-loaded vesicles, while neat Ru(phen)₃ showed >2 LRF after 90 min irradiation.

Furthermore, Figure 7 shows the bar plots of log₁₀ CFU/mL for PA Iso 5 in saline and ASM treated with Ru(phen)₃ loaded vesicles.

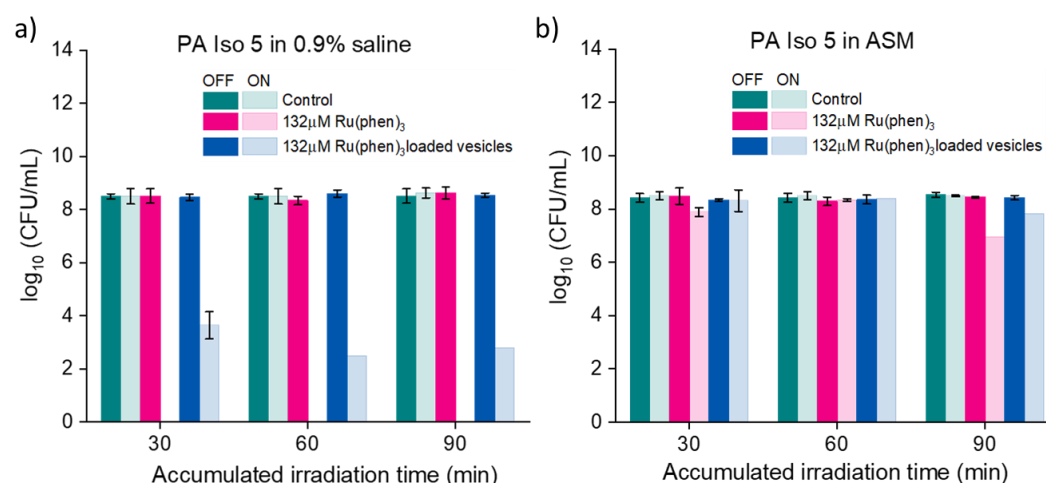


Figure 7: Bar plots of log₁₀ CFU/mL for Ru(phen)₃-loaded vesicles and neat Ru(phen)₃ tested against PA Iso 5 prepared with a) 0.9% saline and b) ASM. The samples were either incubated in the dark (OFF) or irradiated for different irradiation times (ON) at $\lambda = 455$ nm. The error bars correspond to the standard deviation of technical replicates ($n = 3$). No bar corresponds to no countable colonies after 18 h bacterial colony growth.

For PA Iso 5 prepared in saline (Figure 7a), no colonies were observed after 30 min irradiation with neat Ru(phen)₃, whereas Ru(phen)₃-loaded vesicles showed >5 LRF after 30 min which increased to >6 after 60 and 90 min irradiation. In the presence of ASM (Figure 7b), a slight aPDT effect was observed. Ru(phen)₃-loaded vesicles resulted in an LRF of >1, while neat Ru(phen)₃ achieved an LRF of >2 after 90 irradiation, indicating reduced efficacy in ASM compared to saline.

In summary, freshly prepared ASM requires longer irradiation times for bacterial eradication using neat Ru(phen)₃ compared to aged ASM, possibly due to the degradation of organic components over time. Compared to planktonic bacteria in saline, the aPDT efficacy in ASM is significantly reduced by interfering components of the ASM, which reduces singlet oxygen availability. The chelation effect of DTPA in ASM may restrain Ru(phen)₃ and lower its active concentration. Furthermore, DNA and mucin in ASM could interact with Ru(II) complexes via electrostatic interactions, which further compromise aPDT efficacy.²⁹

6.2.2 Investigation of aPDT parameters for biofilms

The following section reports the investigation of aPDT parameters for *P. aeruginosa* biofilms using Ru(phen)₃-loaded vesicles. Initial testing was performed using neat Ru(phen)₃.

Biofilms grown in 96-well plates

PA ATCC and PA Iso 5 biofilms were grown in 96-well plates. After washing the biofilms, 150 µM of Ru(phen)₃ was added and incubated for 15 min followed by 30 min irradiation. The treated biofilms were then collected from wells, serially diluted, and 10 µL of aliquots were plated for CFU counting. Figure 8 shows the bar plot of the log₁₀ CFU/mL of the non-irradiated and irradiated PA ATCC and PA Iso 5 biofilms.

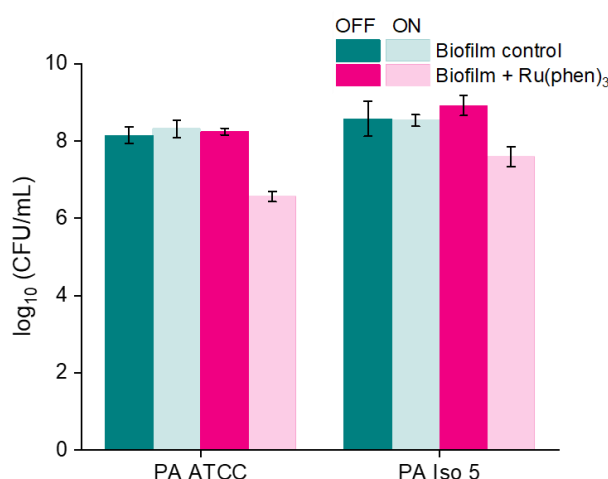


Figure 8: Bar plots of log₁₀ CFU/mL of PA ATCC and PA Iso 5 biofilms treated with 150 µM Ru(phen)₃. The samples were either incubated in the dark (OFF) or irradiated for 30 min (ON) at $\lambda = 455$ nm. The error bars correspond to the standard deviation of technical replicates ($n = 6$).

For both *P. aeruginosa* biofilms, the control samples showed no difference in viable bacterial counts between non-irradiated and irradiated conditions. However, treatment with Ru(phen)₃, followed by irradiation, resulted in a 1.5 log reduction for PA ATCC and a 1 log reduction for PA Iso 5 compared to their respective non-irradiated biofilm controls.

P. aeruginosa biofilms grown in 96-well plates tend to adhere to the bottom surface and extend along the sidewalls of the well. The handling of these biofilms presents challenges due to their susceptibility to detachment during washing or pipetting. Minor mechanical disturbances like pipette suction or washing force can cause uneven biofilm distribution across wells, compromising reproducibility. Additionally, there is a high risk of losing biofilm fragments while removing them from the wells for CFU counting. To address these limitations, biofilms were grown on polycarbonate membranes and evaluated for aPDT efficacy.

Biofilms grown on polycarbonate membranes

For improved experiments, PA ATCC biofilms were grown on polycarbonate membranes. Figure 9a shows the side-view representation of the LB agar with the membrane positioned on top. 100 µL of 10⁶ CFU/mL bacteria suspension was carefully dropped in the center of the membrane. As a control, LB medium was added to the membranes. The agar plates containing inoculated membranes were incubated at 37 °C for 24 h. Figure 9b shows an image of the membrane with LB medium added on top. Nothing grew on the control. Figure 9c shows an image of the membrane following bacterial inoculation. Biofilm formation was clearly observed at the center of the membrane, as concluded from the turbid patchy appearance of a layer on the membrane, which gradually extended outward without leaking or spreading onto the LB agar.



Figure 9: a) Schematic (side view) of bacterial colonies grown on polycarbonate membrane placed on LB agar, b) top view image of the polycarbonate membrane incubated with LB medium, and c) top view image of PA ATCC biofilms grown in LB on the membranes.

To investigate the effects of aPDT on biofilms, the pre-incubation time of Ru(phen)₃ with biofilms was varied to elucidate whether or not this enhances the treatment efficacy.

Effect of pre-incubation time

To investigate the effect of pre-incubation time on aPDT, polycarbonate membranes containing biofilms were transferred to the wells of 6-well plates. 150 µL of 200 µM solution of Ru(phen)₃ in water was added to the biofilms and incubated for either 15 min or 3 h. After

each incubation period, the biofilms were either irradiated for 30 min or kept in the dark. For CFU counting, the treated biofilms (with membranes) were transferred to the 5 mL Eppendorf containing 1 mL PBS. The samples were then sonicated for 5 min and vortexed to dislodge biofilm cells from the membranes. Afterward, serial dilutions were made and 10 μ L aliquots of each dilution were plated on agar. Figure 10 shows the obtained log₁₀ CFU/mL for PA ATCC biofilms treated under these conditions.

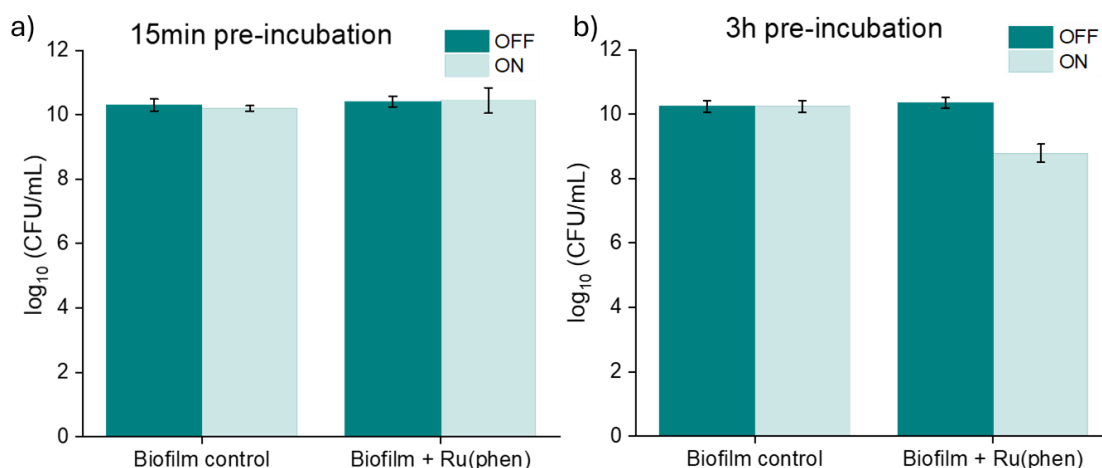


Figure 10: Bar plots of log₁₀ CFU/mL for PA ATCC biofilms grown on polycarbonate membranes with a) 15 min pre-incubation time, and b) 3 h pre-incubation time with 200 μ M Ru(phen)₃. The samples were either incubated in the dark (OFF) or irradiated for 30 min (ON) at $\lambda = 455$ nm. The error bars correspond to the standard deviation of technical replicates ($n = 3$).

Biofilms pre-incubated with Ru(phen)₃ for 15 min showed no reduction in bacterial counts following irradiation under these conditions, both for the biofilm controls or those treated with Ru(phen)₃. However, extending the pre-incubation time to 3 h resulted in a 1.4 log reduction in bacterial counts after irradiation. One may speculate that the EPS matrix of biofilms serves as a major barrier limiting photosensitizer penetration and singlet oxygen diffusion, thereby reducing aPDT efficacy. A longer pre-incubation period allows for increased photosensitizer diffusion into the biofilm, leading to more effective ¹O₂ generation upon irradiation and enhanced bacterial eradication.

aPDT effect of Ru(phen)₃-loaded vesicles against biofilms

The efficacy of aPDT using Ru(phen)₃-loaded vesicles was further evaluated against clinical PA Iso 5 biofilms. Biofilms (24 h, static, 37 °C) were grown on polycarbonate membranes, as elucidated above, and were incubated for 3 h with 150 μ L of 264 μ M neat Ru(phen)₃ or Ru(phen)₃-loaded vesicles. Following incubation, the biofilms were irradiated for different time intervals, followed by serial dilutions of the treated samples and plating of 10 μ L aliquots on agar. Figure 11 shows the log₁₀ CFU/mL of PA Iso 5 biofilms treated at different irradiation intervals.

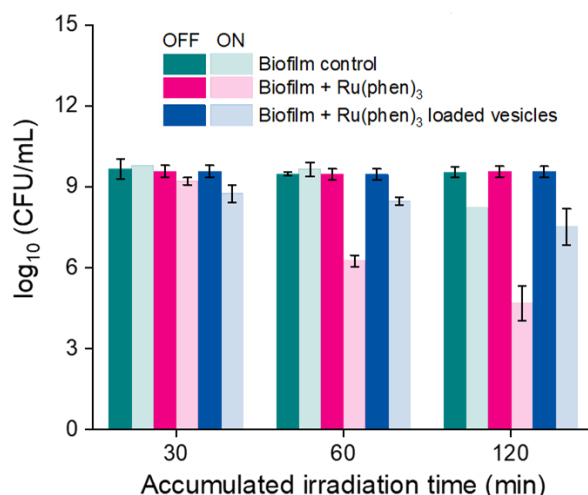


Figure 11: Bar plots of log₁₀ CFU/mL for PA Iso 5 biofilms grown on polycarbonate membranes and subsequently treated with 264 μM neat Ru(phen)₃ and Ru(phen)₃-loaded vesicles. The samples were pre-incubated for 3h and then either incubated in the dark (OFF) or irradiated for different irradiation times (ON) at $\lambda = 455$ nm. The error bars correspond to the standard deviation of technical replicates ($n = 3$).

For biofilm controls, no effect of irradiation was observed after 30 and 60 min irradiation, although a slight decrease in CFU/mL was observed after 120 min irradiation. After 30 min irradiation, neither neat Ru(phen)₃ nor Ru(phen)₃-loaded vesicles caused any aPDT effects. When the irradiation time was increased to 60 min, a 3.5 LRF was observed for neat Ru(phen)₃, and a 1.3 LRF was observed with Ru(phen)₃-loaded vesicles. Extending the irradiation time to 120 min further improved bacterial eradication, with neat Ru(phen)₃ achieving a 4.8 LRF and Ru(phen)₃-loaded vesicles achieving a 2 LRF.

In summary, *P. aeruginosa* biofilms grown on polycarbonate membranes provided a reliable model for aPDT testing compared to those cultured in 96-well plates. As biofilms grown in 96-well plates adhere to the bottom and spread along the sidewalls, making them prone to detachment during washing or pipetting which leads to uneven biofilm distribution and poor reproducibility. Extended pre-incubation time for Ru(phen)₃ and Ru(phen)₃-loaded vesicles improved presumably the penetration of Ru(phen)₃ into the biofilm and enhanced the observed aPDT efficacy.

Comparing these findings with previous reports on aPDT-mediated *P. aeruginosa* biofilm eradication,^{22,29,36} multiple studies have utilized different photosensitizer formulations with high light intensities to achieve some degree of bacterial reduction. Youf et al. demonstrated the eradication of *P. aeruginosa* and *S.aureus* biofilm using neat Ru(phen)₃ under similar irradiation conditions, but only when the Ru(phen)₃ was introduced at the time of biofilm formation, allowing continuous interaction of photosensitizer throughout biofilm development.^{29,29} In contrast, the results in this Chapter show improved aPDT efficacy against mature biofilms using neat Ru(phen)₃, and Ru(phen)₃-loaded vesicles when higher photosensitizer concentration, extended irradiation durations, and longer pre-incubation times

with fully developed biofilms were employed. This highlights the potential of neat Ru(phen)₃ and Ru(phen)₃-loaded vesicles for post-biofilm treatment strategies.

6.3 Conclusion

This Chapter investigated the impact of aPDT parameters to enhance its efficacy against *P. aeruginosa* in ASM and in biofilms grown on polycarbonate membranes. The results highlight the critical influence of biological environments on aPDT performance. Neat Ru(phen)₃ and Ru(phen)₃-loaded vesicles showing distinct behaviors in different medium compositions. In ASM, the aPDT efficacy of Ru(phen)₃-loaded vesicles was significantly lower compared to neat Ru(phen)₃ at similar overall photosensitizer concentrations. This may be attributed to the complex nature of the medium, including both the loaded vesicles and components of ASM, which may hinder the diffusion of ¹O₂ due and/or reduce the availability of Ru(phen)₃ for the aPDT process. Additionally, batch-to-batch variability and stability of ASM over time were found to affect the aPDT outcomes. After 120 min irradiation, *P. aeruginosa* biofilms were successfully eradicated with a 4.8 log reduction with neat 264 μM Ru(phen)₃ and a 2 log reduction was observed with 264 μM Ru(phen)₃-loaded vesicles, implying their potential for post-biofilm formation treatment strategies. Extending the pre-incubation time likely enhanced photosensitizer diffusion into the biofilms and improved the observed aPDT efficacy. These insights provide a foundation for optimizing aPDT for treating *P. aeruginosa* infections in the future, particularly in CF and other biofilm-associated infections.

6.4 Materials and methods

6.4.1 Materials

Samples

Tris(1,10-phenanthroline) ruthenium (II) bis(hexafluorophosphate) (Ru(phen)₃, >98%) was purchased from TCI chemicals (Germany). Mucin from porcine stomach, type II, Egg yolk emulsion, DNA from fish sperm, L-Arginine, Dimethyl sulfoxide (DMSO, 99%), Potassium chloride and Phosphate-buffered saline were purchased from Sigma Aldrich (Germany). L-Serine, L-Valine, L-Proline, L-Methionine, L-Aspartic acid, L-Leucine, Glycine, L-Histidine, L-Glutamine, L-Threonine, L-Isoleucine, L-Phenylalanine, L-Lysine monohydrochloride, L-Asparagine monohydrate, L-Tyrosine, L-Cysteine, L-Glutamic acid, L-Alanine, Sodium chloride, Potassium hydroxide and Diethylenetriaminepentaacetic acid were purchased from Carl Roth (Germany). Singlet Oxygen Sensor Green SOSG (Thermo Fischer Scientific, Eugene, OR, USA), Methanol (>99.8%, VWR, Germany), Tris buffer 100 mM pH 7.4 (Millipore Sigma, Germany), L(-)-Tryptophan (PanReac AppliChem, Germany), Tris 1 M buffer solution

pH 8.5 (Thermo scientific, Germany), Lysogeny broth (LB, Luria/Miller: 10g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7.0 ± 0.2, Carl Roth GmbH, Germany), LB agar (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 15 g/L agar-agar, pH 7.0 ± 0.2 Carl Roth, Germany), Nucleopore track-etch membrane (VWR, Germany), sterile water and 0.9% saline (Versylene Fresenius, Germany), transparent flat-bottom 96 well plate (Sarstedt), and cell culture 96 black half area μ -clear well plate (Greiner Bio-One, Germany) were purchased from the mentioned suppliers. Milli-Q water (for preparing LB medium and LB agar plates) was drawn from a Millipore Direct Q8 system with a resistivity of 18.2 M Ω cm (Millipore advantage A10 system, Schwalbach, with Millimark Express 40 filter, Merck, Germany).

Bacterial strains

Pseudomonas aeruginosa lab strain ATCC 19660 (isolated from samples taken from a sepsis patient in Lima, Peru, and provided by ATCC) here noted as PA ATCC and *P. aeruginosa* cystic fibrosis patient strain CH2678 (isolated from sputum, isolated at Charité Berlin, provided by Prof. Susanne Häußler, Helmholtz Centre for Infection Research, Braunschweig, Germany), denoted here as PA Iso 5, were used in this Chapter.

Sample preparation

Synthesis of Ru(phen)₃ solution

The 5 mM stock solution of Ru(phen)₃ was prepared in DMSO. The desired final solution concentrations were prepared in sterile water for aPDT and singlet oxygen detection.

Preparation of ASM

ASM was prepared according to Kirchner et al.⁵ Briefly, 1.00 g DNA from fish sperm was added slowly to 62.5 mL sterile water and stirred overnight at room temperature. Separately, 1.25 g mucin from porcine stomach (type II) was added slowly to 62.5 mL sterile water and stirred until the mucin dissolved completely. The 0.06 g of each essential and non-essential L-amino acid, except L-tyrosine and L-cysteine, was dissolved in 25 mL sterile water. Separately, 0.06 g of L-cysteine was dissolved in 6.25 mL of 0.5 M potassium hydroxide, and 0.06 g of L-tyrosine was dissolved in 6.25 mL sterile water. Separately, 1.47 mg diethylenetriaminepentaacetic acid (DTPA), 1.25 g NaCl, and 0.55 g of KCl were dissolved in 25 mL sterile water. After ensuring complete mixing, DNA, mucin, L-amino acids, DTPA, NaCl, and KCl solutions were combined in a 250 mL bottle. The pH was adjusted to 6.9 with 1M Tris (pH 8.5), and the volume was brought to 250 mL with sterile water. Autoclaving conditions without pressure were used for sterilization. The solution was heated for 15 min at 121 °C. Further steps were done in the sterile hood. After cooling, 1.25 mL of egg yolk emulsion was added. The prepared ASM was stored at 4 °C in the dark. To check the sterility of the prepared ASM, 50 μ L of ASM was plated on LB agar and incubated for 24 h at 37 °C. No bacterial colonies were spotted.

6.4.2 Methods

Fluorescence spectroscopy

The fluorescence intensity was measured using a microplate reader (Tecan SAFIRE, Tecan, Switzerland). The measurements were recorded using cell culture 96 black half area μ -clear well plates with fluorescence top reading mode at $\lambda_{\text{ex}} = 504$ nm and $\lambda_{\text{em}} = 528$ nm.

Irradiation experiments

A homemade LED irradiation setup was used to perform the irradiation experiments (as shown in Chapter 5, Figure 27). The setup includes two panels of 225 blue LEDs (14 W, $\lambda = 450$ -460 nm, HQRP LED plant grow panel lamp system, US) facing each other at adjustable distances. The panels were placed in such a way on top and bottom to have an exposure area in the center to achieve irradiation from both sides of the well plate. The irradiation intensity at the exposure area was adjusted to 2.7 - 3.0 mW/cm², as measured using a PM 160 wireless handled power meter (Thorlabs, Bergkirchen, Germany).

For irradiation, the sample well plate was placed in the exposure area and was irradiated at room temperature for different irradiation times. To assess the effect of irradiation, treated samples were compared with samples not exposed to light. In this Chapter, these conditions are referred to as OFF' (non-irradiated samples) and 'ON' (irradiated samples).

Detection of singlet oxygen production

To detect the production of singlet oxygen, the fluorescent singlet oxygen sensor green SOSG probe was used.ref. In all experiments, 90 μ L of the Ru(phen)₃ samples were mixed with 10 μ L of 100 μ M SOSG probe (final SOSG concentration: 10 μ M). The samples were incubated in the dark for 15 min and then irradiation was performed. The SOSG fluorescence signal for irradiated and non-irradiated samples was measured at an excitation of 504 nm and emission at 528 nm. For both the SOSG testing and aPDT experiments, Ru(phen)₃ samples were mixed in 1:1 ratio with the studied different mediums to have their final concentrations to be $\sim$ half of the undiluted photosensitizer.

aPDT effect on planktonic bacteria

Overnight cultures were prepared by taking single colonies of each bacterial strain in 5.0 mL LB followed by incubating at 37 °C for 18 h while shaking at 200 rpm. Then 1.0 mL of the cultures were centrifuged at 10,000 rpm for 5 min. The supernatant was discarded and the pellet was resuspended in 0.9% NaCl or ASM. The OD at 600 nm of each bacteria solution was adjusted to 0.6, corresponding to $\sim 10^8$ CFU/mL. For some experiments, the starting inoculum concentrations were $\sim 10^6$ prepared by 100x dilution in 0.9% NaCl or ASM. Afterward, 25 μ L of bacteria solutions were distributed in a 96 well-plate containing 25 μ L of Ru(phen)₃ solutions. Each sample condition was performed in technical triplicate (3 wells), separately for

irradiated and non-irradiated samples. The samples were incubated for 15 min at room temperature and then the irradiation was performed. To evaluate the aPDT effect, 10 μ L of each technical replicate (well) of the irradiated and non-irradiated undiluted and serially diluted samples were spotted three times on agar plates (30 μ L in total) that results in a detection limit of the method of 33 CFU/mL. The plates were incubated overnight at 37 °C, and the next day bacterial colonies were counted for CFU/mL calculations of all sample conditions. The log₁₀ CFU/mL and the LRF were then calculated. For the LRF the respective non-irradiated bacteria suspension was taken as the control.

aPDT effect in biofilms

Biofilms grown in 96 well plate

Overnight culture of PA ATCC and PA Iso 5 was prepared by mixing one colony of each bacteria in 5 mL LB and placed on a shaker at 200 rpm, 37 °C for 18h. The biofilms were established in 96-well plates by adding 100 μ L of bacterial inoculums of 10⁶ CFU/mL. The well plate was covered with a breathable polyurethane membrane. To maintain the humidity, the well plate was placed in a humid chamber. The plates were incubated for 24 h at 37 °C under static conditions.

The 100 μ L of spent media, planktonic cells and unadhered bacteria was carefully aspirated from the wells. The adhered biofilms were washed by adding 150 μ L fresh sterile LB media. Then the well plate was placed on the shaker at 120 rpm for 5 min. The rinsing media was aspirated and discarded to a waste container. The washing was repeated with LB for two more times followed by one time rinsing with 150 μ L 0.9% saline. The liquid was aspirated as much as possible out of each biofilm-containing well using a multichannel pipette. For treatment, 55 μ L of 0.9% saline was added on top of the biofilm and 55 μ L of 300 μ M Ru(phen)₃. The well plates were incubated in the dark for 15 min. One well plate was irradiated for a total of 30 min while the other well plate was kept in the dark. For CFU counting, the biofilm samples were collected by vigorously mixing the solution in each well using a multichannel pipette, also around the side walls of the wells. 10-fold serial dilutions of the collected samples were done and 10 μ L of each dilution was spotted on square agar plates.

Biofilms grown on polycarbonate membrane

The biofilms growth on polycarbonate membranes were adapted from Meritt et al.³⁷ Both sides of the membranes were sterilized for 10 min under UV light (Benchtop UV Transilluminator, 250 V) before being placed on LB agar plates with the shiny side facing upward. 100 μ L of bacteria suspension (PA ATCC and PA Iso 5, 10⁶ CFU/mL in LB) were carefully dropped in the center of the membrane. As a control, LB medium was added to the membranes. The agar plates containing inoculated membranes were incubated at 37 °C for 24 h. Polycarbonate membranes containing biofilms were then transferred to the wells of 6-well plates. 150 μ L of

200 µM photosensitizer was added to the biofilms and incubated for 15 min or 3 h. After each incubation period, the biofilms were either irradiated for 30 min or were kept in the dark. For CFU counting, the treated biofilms (with membranes) were transferred to the 5 mL Eppendorf containing 1 mL PBS. The samples were then sonicated for 5 min and vortexed to dislodge biofilm cells from the membranes. Afterward, serial dilutions were made and 10 µL aliquots of each dilution were plated on agar.

6.5 References

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7 aPDT against clinically relevant isolates

This Chapter reports the results of antimicrobial photodynamic therapy (aPDT) treatment using neat Ru(phen)₃ and Ru(phen)₃-loaded vesicles against clinically relevant bacteria, including *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Staphylococcus aureus* (*S. aureus*) isolates collected from Cystic Fibrosis (CF) patients. Furthermore, aPDT of Ru(phen)₃ was tested against pathogenic *Escherichia coli*.

7.1 Introduction and objectives

As described in Chapter 2 and 6, *P. aeruginosa* is among the most prevalent bacterial species colonizing the lungs of individuals with cystic fibrosis (CF) and is a major contributor to poor clinical outcomes due to the difficulty in its eradication.¹ *S. aureus* is another dominant pathogen in CF lungs, and it is frequently co-detected with *P. aeruginosa* in approximately 20-50% of patients.^{2,3} The co-existence of these pathogens further complicates treatment, especially considering their ability to form robust biofilms.^{4,5} These biofilms confer significant protection against antibiotics and the host immune system,⁶ thereby hindering effective bacterial eradication. Moreover, the emergence of multi-drug resistant (MDR) organisms, many of which are listed as priority pathogens by the World Health Organization (WHO)⁷, is increasingly reported in CF populations^{8,9} further underscoring the urgency for alternative antimicrobial strategies.

aPDT has gained attention as a promising adjunctive therapy for the treatment of resistant bacterial infections in CF. Several studies have reported the efficacy of aPDT in inactivating both *P. aeruginosa* and *S. aureus*, including antibiotic-resistant strains and biofilm-embedded populations,¹⁰ thereby enhancing the susceptibility of these bacteria to conventional antibiotics and host immune mechanisms.¹¹ In the present Chapter, aPDT effects of Ru(phen)₃ loaded vesicles and neat Ru(phen)₃ were tested against different *P. aeruginosa* and *S. aureus* CF isolates. The aPDT effect of neat Ru(phen)₃ was further tested against pathogenic *Escherichia coli*.

7.2 Results and discussion

The parameters for aPDT were investigated using neat Ru(phen)₃ by varying its concentrations and irradiation times. Following Ru(phen)₃-loaded PEG₁₁₄-*b*-PLA₅₀₀ vesicles, containing higher Ru(phen)₃ concentrations in suspensions, were evaluated for their efficacy against a series of *P. aeruginosa* and *S. aureus* CF isolates. The Ru(phen)₃-loaded PEG₁₁₄-*b*-PLA₅₀₀ vesicles used in this Chapter were provided by M. Sc. Max Müller. The aPDT efficacy of neat Ru(phen)₃ was also tested against pathogenic *E. coli*.

7.2.1 aPDT efficacy of Ru(phen)₃-loaded vesicles against *P. aeruginosa* CF isolates

The different PA CF isolates^{12,13} used for aPDT experiments are listed in Table 1, including information on the origin, the infection type, and the resistance of these isolates towards different antibiotics.

Table 1: The *P. aeruginosa* CF isolates used in this Chapter. Where rt: respiratory tract, nd: not defined, TOB: Tobramycin, CIP: Ciprofloxacin, MEM: Meropenem, CAZ: Ceftazidime, COL: Colistin, S: Susceptible, I: Intermediate, R: Resistant.

PA CF Isolate	Abbreviation used in text	Type of infection	Origin	Resistance towards antibiotics				
				TOB	CIP	MEM	CAZ	COL
CF592	Iso 1	rt	University Hospital Essen	S	S	I	R	S
CH2678	Iso 5	rt	Charité Berlin	S	R	S	S	R
M70565254	Iso 28	rt	University Hospital Münster	S	S	R	R	R
MHH0426	Iso 31	nd/other	Hannover Medical School	S	I	S	S	S
MHH0927	Iso 32	nd/other		S	S	S	S	R
MHH15204	Iso 36	rt		S	I	R	S	S
MHH16530	Iso 38	rt		S	S	R	R	S
MHH17546	Iso 43	rt		R	R	R	R	S
MHH17783	Iso 45	rt		S	S	R	R	S
MHH1883	Iso 46	nd/other		S	S	S	I	S
MHH2419	Iso 47	nd/other		S	S	S	S	S

Before testing aPDT with Ru(phen)₃ loaded vesicles, the Ru(phen)₃ concentrations were varied to find the optimum PS concentration for aPDT.

aPDT against PA CF isolates with varied Ru(phen)₃ concentrations

The bacterial suspensions of PA CF isolates were prepared by centrifuging overnight cultures to obtain pellets, which were then resuspended in 0.9% saline. Bacteria suspension were incubated with Ru(phen)₃ solution for 15 min (final concentrations of Ru(phen)₃ 25, 12.5, and 6.25 µM) followed by irradiation for 10 min. Serial dilutions of the treated samples were performed and 10 µL aliquots of each dilution were plated onto LB agar for non-irradiated and irradiated samples. The CFU/mL was calculated and the log reduction in bacterial colonies

was then compared to non-irradiated bacteria controls. The obtained log reduction factor (LRF) values for different PS concentrations are given in Figure 1.

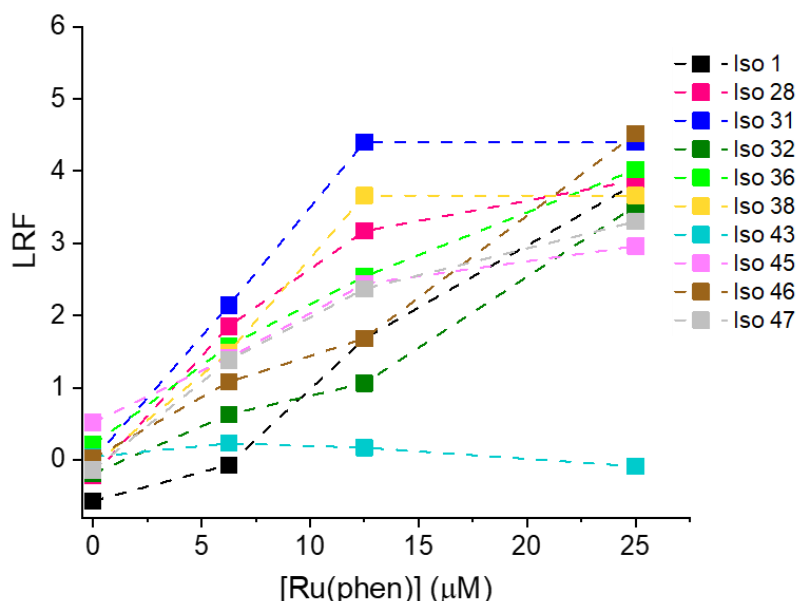


Figure 1: Log reduction factor for different PA CF isolates in 0.9% saline treated with different Ru(phen)_3 concentrations. The samples were irradiated for 10 min at $\lambda = 455$ nm.

At a Ru(phen)_3 concentration of 6.25 μM , no bactericidal effect (i.e. a ≥ 3 log₁₀ reduction in CFU) was observed for any of the tested isolates. When the Ru(phen)_3 concentration was doubled, bactericidal effects were observed only in Iso 31 (sensitive to 4 out of 5 antibiotics), Iso 38 (sensitive to 3 out of 5 antibiotics), and Iso 28 (sensitive to 2 out of 5 antibiotics) (Table 1). At a Ru(phen)_3 concentration of 25 μM , all tested isolates except for Iso 43 showed bactericidal response. Iso 43, which is the most antibiotic-resistant among the tested strains, did not show aPDT effect at the tested PS concentrations.

aPDT with Ru(phen)_3 -loaded vesicles

Bacterial suspensions of PA CF isolates were prepared in 0.9% saline. Ru(phen)_3 -loaded vesicles and neat Ru(phen)_3 solutions were incubated with bacteria suspensions for 15 min (final concentrations of Ru(phen)_3 : 25 μM) followed by irradiation for 30 min. To assess bacterial viability, serial dilutions of the treated samples were performed and 10 μL aliquots of each dilution were plated onto LB agar for non-irradiated and irradiated samples. The CFU/mL was calculated, and the log reduction in bacterial colonies was then calculated compared to respective bacteria controls in the dark. The obtained log reduction factor (LRF) values are given in Figure 2.

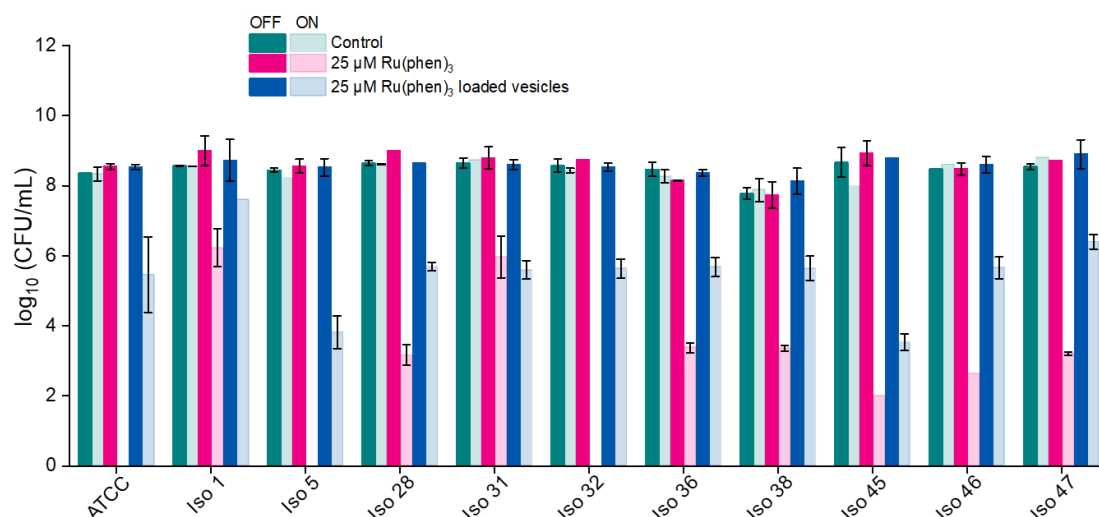


Figure 2: Bar plots of $\log_{10}$ CFU/mL for different PA CF isolates in 0.9% saline treated with 25 μ M neat Ru(phen)₃ and Ru(phen)₃-loaded vesicles. The samples were either incubated in the dark (OFF) or irradiated for 30 min (ON) at $\lambda = 455$ nm. The error bars correspond to the standard deviation of technical replicates ($n = 3$).

No reduction in CFU counts was observed in the bacterial controls after irradiation. Treatment with neat Ru(phen)₃ resulted in a noticeable reduction in CFU for all bacteria suspensions, with complete eradication (i.e., no countable colonies were in the case of ATCC, Iso 5, and Iso 32). With Ru(phen)₃-loaded vesicles, smaller CFU counts were observed after irradiation compared to the corresponding non-irradiated samples. The resulting LRF values obtained after irradiation compared to the respective bacteria control in dark are given in Table 2. The detection limits of colony counting were included in LRF calculations.

Table 2: LRF for the *P. aeruginosa* CF isolate treated with 25 μ M Ru(phen)₃-loaded vesicles and neat Ru(phen)₃.

PA Isolate	LRF after 30 min irradiation	
	Ru(phen) ₃	Ru(phen) ₃ -loaded vesicles
ATCC	6.84	2.90
Iso 1	2.34	0.97
Iso 5	6.92	4.62
Iso 28	5.48	2.95
Iso 31	2.68	3.06
Iso 32	7.05	2.94
Iso 36	5.09	2.79
Iso 38	4.42	2.14
Iso 45	6.66	5.13
Iso 46	5.84	2.82
Iso 47	5.33	2.15

The effect of neat $\text{Ru}(\text{phen})_3$ was bactericidal (≥ 3 log reduction), including 4 isolates with LRF >6 . For $\text{Ru}(\text{phen})_3$ -loaded vesicles, over 99% of the bacteria were killed (>2 log reduction) with two isolates showing >4 LRF, except for Iso 1. Current experimental conditions can eradicate the different CF isolates with variable resistance. For improved aPDT efficacy with $\text{Ru}(\text{phen})_3$ -loaded vesicles, a higher $\text{Ru}(\text{phen})_3$ concentration is required.

Among all the tested isolates, Iso 43 was the only one that did not show any aPDT effect (Figure 1). Hence, Iso 43 was irradiated for longer times, 30 min and 60 min. Figure 3 shows the resulting CFU/mL of Iso 43 treated with neat $\text{Ru}(\text{phen})_3$ and $\text{Ru}(\text{phen})_3$ -loaded vesicles.

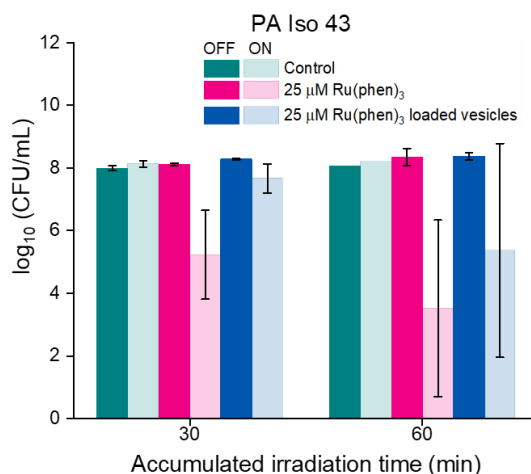


Figure 3: Bar plots of log₁₀ CFU/mL for PA CF Iso 43 in 0.9% saline treated with $\text{Ru}(\text{phen})_3$ -loaded vesicles and neat $\text{Ru}(\text{phen})_3$. The samples were either incubated in the dark (OFF) or irradiated for different irradiation times (ON) at $\lambda = 455$ nm. The error bars correspond to the standard deviation of biological replicates ($N=2$, $n = 6$).

For bacteria controls, no change in CFU was observed after irradiation. In the case of both neat $\text{Ru}(\text{phen})_3$ and $\text{Ru}(\text{phen})_3$ -loaded vesicles, longer irradiation time resulted in decreasing CFU/mL compared to non-irradiated samples. The large error bars in the plot indicated the deviation between separate biological experiments.

With neat $\text{Ru}(\text{phen})_3$, 2.76 LRF was observed after 30 min and 4.54 LRF was observed after 60min. With $\text{Ru}(\text{phen})_3$ -loaded vesicles, 0.32 LRF was observed after 30 min and 2.68 LRF was observed after 60 min irradiation. Despite the deviations, the results indicated that it is possible to eradicate this highly resistant Iso 43 using $\text{Ru}(\text{phen})_3$, required better understanding of bacteria interaction with the photosensitizer.

The higher aPDT efficacy of neat $\text{Ru}(\text{phen})_3$ compared to $\text{Ru}(\text{phen})_3$ -loaded vesicles may be attributed to its higher bioavailability and/or enhanced interactions with bacterial cells, leading to more efficient $^1\text{O}_2$ generation near the bacterial targets. Whereas encapsulation of $\text{Ru}(\text{phen})_3$ within vesicles can hinder its release, restrict $^1\text{O}_2$ diffusion and reduce interactions with bacterial cells, thereby limiting overall aPDT performance.

7.2.2 aPDT of Ru(phen)₃-loaded vesicles against *S. aureus* CF isolates

S. aureus is another clinically relevant pathogen commonly associated with CF infections. The four CF isolates used in this Chapter are given in Table 3. Before checking aPDT efficacy with Ru(phen)₃-loaded vesicles, adequate aPDT parameters were established using neat Ru(phen)₃.

Table 3: *S. aureus* CF isolates used in this Chapter.¹⁴

SA CF Isolate	Abbreviation used in text	Blood agar phenotype
t11	Iso 1	non- mucoid
t13	Iso 2	mucoid
DIV-225	Iso 225	non-mucoid
DIV-208	Iso 208	non-mucoid

Investigation of aPDT parameters against *S. aureus*

Bacterial suspensions of SA CF isolates were prepared by centrifuging overnight cultures to obtain pellets, which were then resuspended in 0.9% saline. The Ru(phen)₃ solutions with different concentrations were mixed with the bacterial suspensions (10^6 CFU/mL) in a 1:1 ratio. Ru(phen)₃ was incubated with bacteria suspensions for 15 min (final concentrations of Ru(phen)₃: 25 and 50 μ M) followed by irradiation for 10, 20, and 30 min. Afterwards, serial dilutions of the treated samples were performed, and 10 μ L aliquots of each dilution were plated onto LB agar for non-irradiated and irradiated samples (Figure 4).

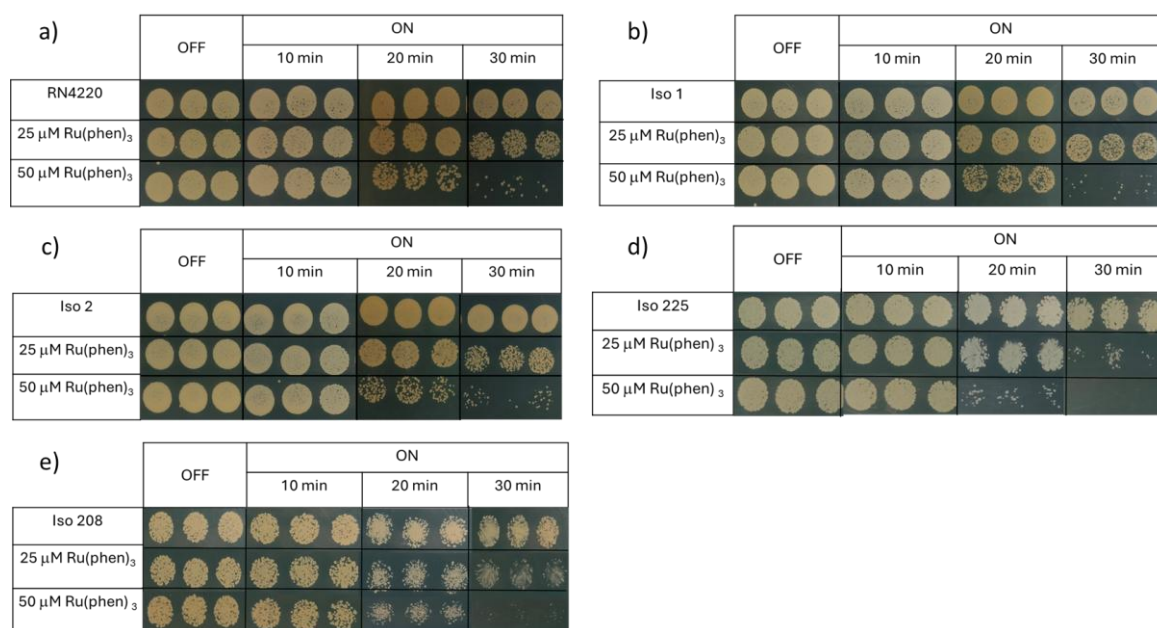


Figure 4: Spotting colonies (after 20 h growth) of a) lab strain RN4220 and b-e) different SA CF isolates in 0.9% saline treated with 25 μ M and 50 μ M Ru(phen)₃. The samples were either incubated in the dark (OFF) or irradiated for different times (ON) at $\lambda = 455$ nm.

The non-irradiated samples showed similar bacterial growth to their corresponding bacterial controls. In the presence of 25 μM Ru(phen)₃, 30 min irradiation was required to observe any decrease in colony growth. For 50 μM Ru(phen)₃, a decrease in bacterial colonies was observed after 20 min irradiation, with improved aPDT after 30 min irradiation. No spotted colonies were observed for Iso 225 after 30 min irradiation when treated with 50 μM Ru(phen)₃.

The log₁₀ CFU/mL values were then calculated for each experimental condition; the resulting bar plots are shown in Figure 5.

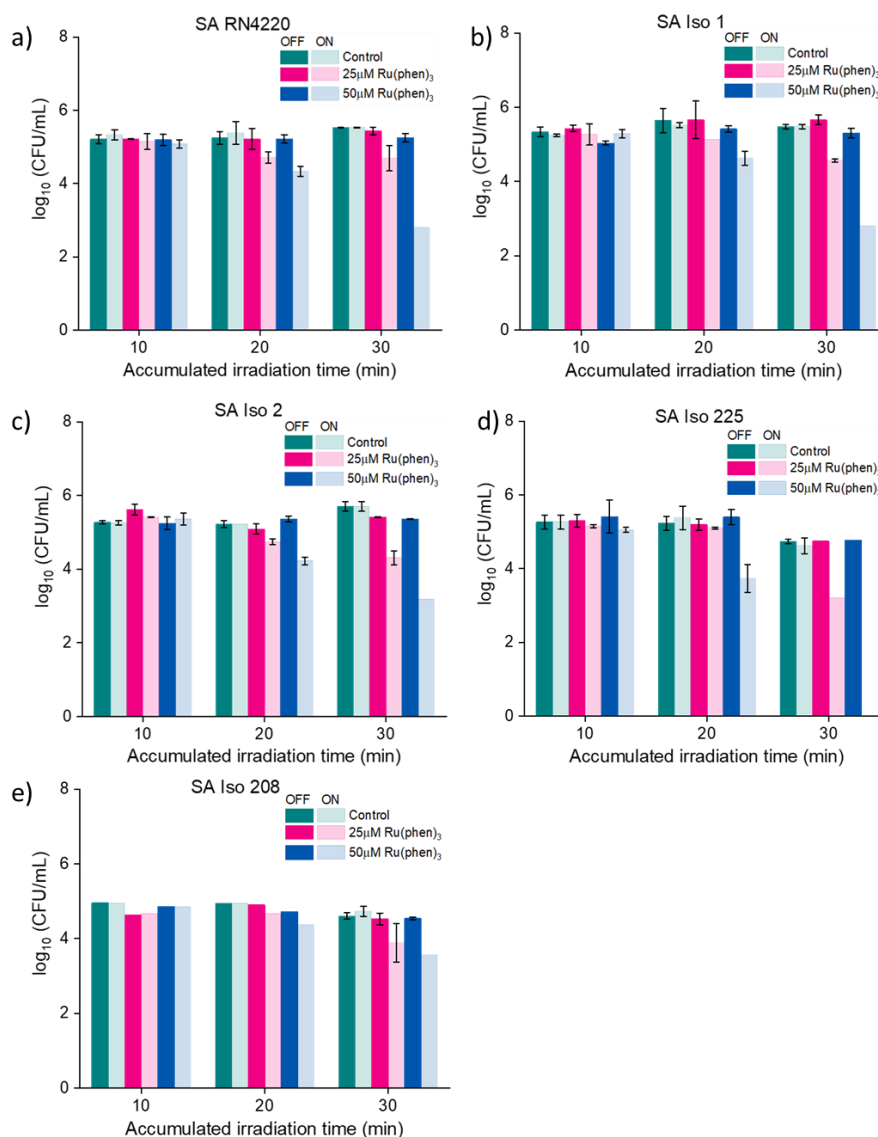


Figure 5: Bar plots of log₁₀ CFU/mL for a) lab strain RN4220 and b-e) different SA CF isolates in 0.9% saline treated with 25 μM and 50 μM Ru(phen)₃. The samples were either incubated in the dark (OFF) or irradiated for different times (ON) at $\lambda = 455$ nm. The error bars correspond to the standard deviation of technical replicates ($n = 3$).

After 10 min irradiation, no change in CFU was observed in either case. Increasing PS concentration and irradiation time improved the bacteria eradication. Comparing the effects for 50 μM PS after 30 min irradiation, 2.73 LRF was observed for RN4220, 2.76 LRF for Iso

1, 2.52 LRF for Iso 2, 3.22 LRF for Iso 225, and 1.04 for Iso 208. The LRF values were calculated considering the detection limits of the experiment.

aPDT with $Ru(phen)_3$ -loaded vesicles against *S. aureus*

The final $Ru(phen)_3$ concentration of 50 μM was chosen to test aPDT with $Ru(phen)_3$ -loaded vesicles. The $Ru(phen)_3$ -loaded vesicles and neat $Ru(phen)_3$ solutions were mixed with the bacterial suspensions (10^8 CFU/mL) in a 1:1 ratio. The $Ru(phen)_3$ solutions were incubated with bacteria suspension for 15 min (final $Ru(phen)_3$ concentrations: 50 μM) followed by irradiation for 30 and 45 min. Afterwards, serial dilutions of the treated samples were performed, and 10 μL aliquots of each dilution were plated onto LB agar for non-irradiated and irradiated samples. The $\log_{10}$ CFU/mL was then calculated, and the resulting bar plots are shown in Figure 6.

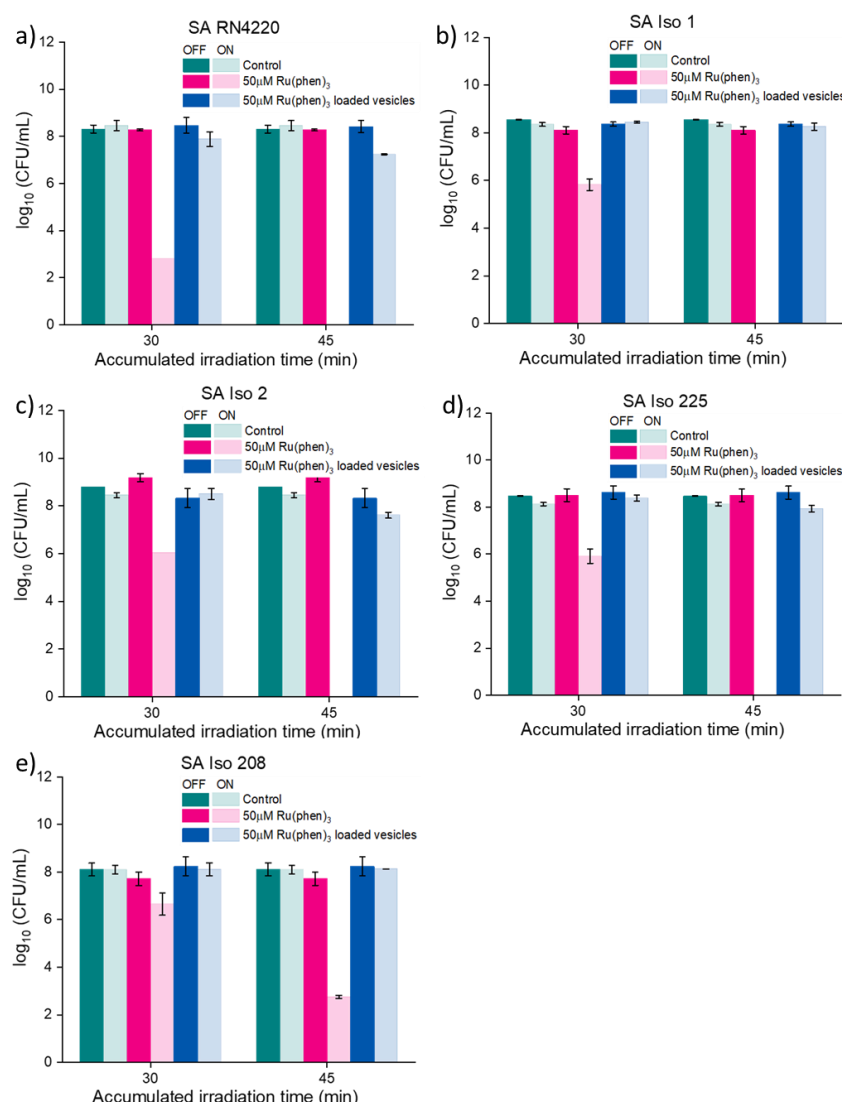


Figure 6: Bar plots of $\log_{10}$ CFU/mL for a) lab strain RN4220 and b-e) different SA CF isolates in 0.9% saline treated with 50 μM $Ru(phen)_3$ -loaded vesicles and neat $Ru(phen)_3$. The samples were either incubated in the dark (OFF) or irradiated for different times (ON) at $\lambda = 455$ nm. The error bars correspond to the standard deviation of technical replicates ($n = 3$).

For bacteria controls, no change in CFU values was observed. The reduction in CFUs was observed only when treated with neat Ru(phen)₃. For 50 µM Ru(phen)₃-loaded vesicles, no aPDT effect was observed. The resulting LRF values obtained are given in Table 4. The detection limits of the bacterial counting were included in the LRF calculations.

Table 4: LRF for the *S. aureus* CF isolate treated with 50 µM Ru(phen)₃-loaded vesicles and neat Ru(phen)₃.

SA Isolates	50 µM Ru(phen) ₃		Ru(phen) ₃ -loaded vesicles [50 µM] in suspension	
	Irradiation time (min)		Irradiation time (min)	
	30	45	30	45
RN4220	5.50	6.78	0.42	1.07
Iso 1	2.72	7.03	0.11	0.29
Iso 2	2.73	7.25	0.28	1.17
Iso 225	2.55	6.93	0.08	0.54
Iso 208	1.46	5.36	0.00	-0.02

For bacterial suspensions with neat Ru(phen)₃ after 45 min irradiation, a >6 LRF was observed for lab strain RN4220 and CF iso 225, while CF Iso 1 and Iso 2 achieved a >7 LRF, and a >5 LRF was observed for Iso 208. When bacterial suspensions were treated with Ru(phen)₃-loaded vesicles, no aPDT effect was observed, except for RN4220 and Iso 2, showing >1 LRF after 45 min irradiation.

To improve the aPDT efficacy with Ru(phen)₃-loaded vesicles, the vesicles with a higher Ru(phen)₃ concentration (final concentration in suspension = 135 µM) were tested with the SA CF isolates. The irradiation time was also increased to 60 min for improved effects. For control, neat Ru(phen)₃ was also tested with the same final concentration as Ru(phen)₃-loaded vesicles. The unloaded vesicles were also tested, and Figure 7 shows the corresponding CFU/mL values obtained after the treatment of different SA bacterial suspensions.

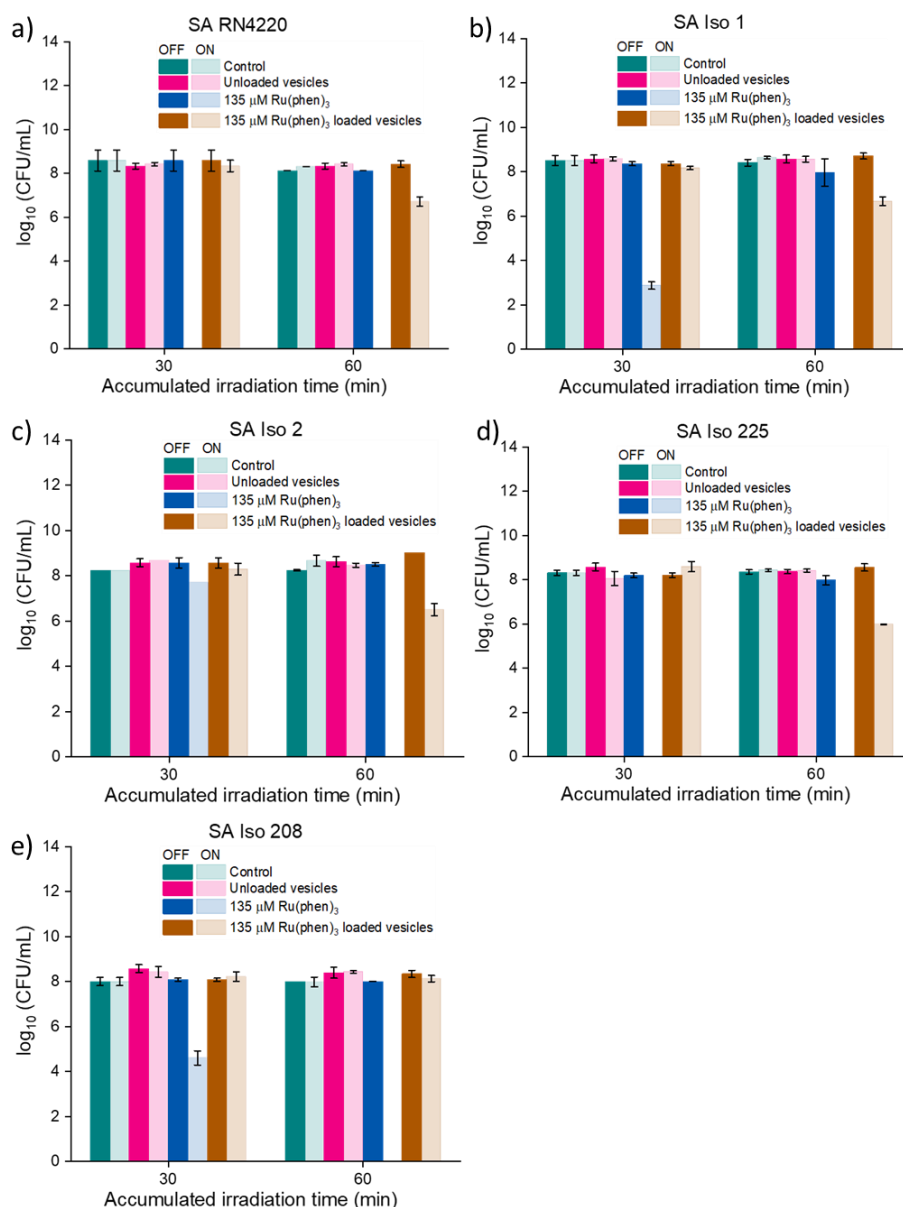


Figure 7: Bar plots of log₁₀ CFU/mL for a) lab strain RN4220 and b-e) different SA CF isolates in 0.9% saline treated with 135 μM Ru(phen)₃-loaded vesicles and neat Ru(phen)₃. The samples were either incubated in the dark (OFF) or irradiated for different times (ON) at $\lambda = 455$ nm. The error bars correspond to the standard deviation of technical replicates ($n = 3$).

The bacterial controls and the unloaded vesicles did not show any change in CFU after irradiation compared to their non-irradiated samples. With neat Ru(phen)₃, no bacterial growth was observed for RN4220 and Iso 225 after 30 min irradiation, while 60 min irradiation was required for Iso 1, Iso 225, and Iso 208 to achieve complete bacterial eradication. For Ru(phen)₃-loaded vesicles, no change in CFU was observed after 30 min irradiation. The slight aPDT effects were observed after 60 min. Table 5 gives the resulting LRF after considering the detection limits of the experiment.

Table 5: LRF for the *S. aureus* CF isolate treated with 135 μM Ru(phen)₃-loaded vesicles and neat Ru(phen)₃.

SA Isolates	135 μM Ru(phen) ₃		Ru(phen) ₃ -loaded vesicles [135 μM] in suspension	
	Irradiation time (min)		Irradiation time (min)	
	30	60	30	60
RN4220	7.06	6.59	0.24	1.40
Iso 1	5.63	6.89	0.34	1.74
Iso 2	0.52	6.72	-0.07	1.73
Iso 225	6.79	6.83	-0.28	2.37
Iso 208	3.39	6.48	-0.22	-0.12

After 60 min irradiation, neat Ru(phen)₃ achieved a >6 LRF for each bacterial suspension. However, for Ru(phen)₃-loaded vesicles, a 1.4 LRF was observed in the case of lab strain RN4220, 1.74 for Iso 1, 1.73 for Iso 2, and 2.37 for Iso 225. No aPDT effect observed for Iso 208 in the presence of Ru(phen)₃-loaded vesicles. The negative LRF values observed are not significant. Comparing these LRF values to the LRF values for Ru(phen)₃-loaded vesicles in Table 4, 60 min irradiation and higher Ru(phen)₃ concentration were required to observe improvement in the aPDT efficacy against *S. aureus* isolates.

In summary, neat 50 μM Ru(phen)₃ after 30 min irradiation showed >2.5 log reduction for RN4220, Iso 1, and Iso 2, >3 LRF for Iso 225, and >1 for Iso 208. Extending the irradiation time to 45 min significantly enhanced the aPDT efficacy, yielding >6 LRF for RN4220, Iso 1, Iso 2, and Iso 225, and >5 LRF for Iso 208.

By contrast, when Ru(phen)₃-loaded vesicles were tested under same irradiation conditions (50 μM , and irradiation 45 min), no significant aPDT effect was observed, except for minor reductions (LRF >1) in RN4220 and Iso 2. To improve the efficacy of Ru(phen)₃-loaded vesicles, the final Ru(phen)₃ concentration in suspension was increased to 135 μM , and the irradiation time was extended to 60 min. Under these conditions, neat Ru(phen)₃ achieved complete bacterial eradication (>6 LRF) for all tested isolates. However, Ru(phen)₃-loaded vesicles showed only moderate aPDT activity, with maximum LRF values of 2.37 for Iso 225, 1.74 for Iso 1, and 1.73 for Iso 2. No aPDT effect was observed for Iso 208.

These findings indicate that encapsulation significantly reduced the bioavailability and effectiveness of the Ru(phen)₃, necessitating higher concentrations and longer irradiation to achieve moderate antibacterial effects. This highlights the limitations of vesicle-based delivery systems for Ru(phen)₃ in aPDT applications against *S. aureus*.

Effect of varied pre-incubation time on aPDT efficacy

Until now, the aPDT experiments were studied at a 15 min pre-incubation time of the Ru(phen)₃ with the bacteria suspension. To investigate if this pre-incubation time affects the aPDT results, the bacteria suspensions of PA ATCC (lab strain), PA Iso 5 (CF isolate), and SA RN4220 (lab strain) were incubated with neat Ru(phen)₃ and Ru(phen)₃-loaded vesicles for 3 h before the irradiation step. For control, the samples were pre-incubated for 15 min. The samples were then irradiated for 30 min or kept in the dark. The 10 µL aliquots of the samples were plated on agar, and the resulting CFU/mL values are shown in Figure 8.

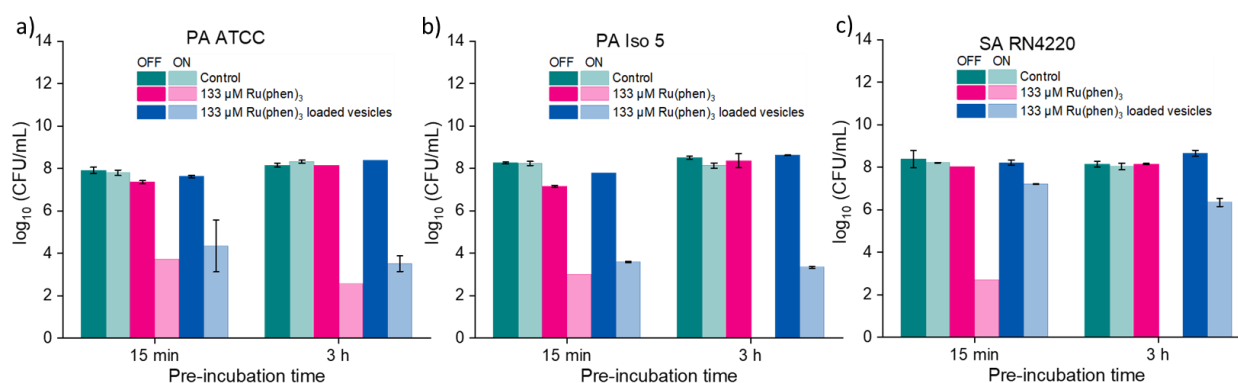


Figure 8: Bar plots of log₁₀ CFU/mL for a) PA ATCC, b) PA Iso 5, and c) SA RN4220 in 0.9% saline treated with 133 µM Ru(phen)₃-loaded vesicles studied at different pre-incubation times. The samples were either incubated in the dark (OFF) or irradiated for 30 min (ON) at $\lambda = 455$ nm. The error bars correspond to the standard deviation of technical replicates ($n = 3$).

The different pre-incubation times and the light irradiation did not affect the CFU values for bacteria controls. For each bacterial suspension, the neat Ru(phen)₃ and Ru(phen)₃-loaded vesicles showed changes in the CFU counts when compared between 15 min and 3 h pre-incubation time. The log reductions in the bacterial counts for each experimental condition are compiled in Table 6.

Table 6: LRF for the different bacteria suspensions treated with 133 µM Ru(phen)₃-loaded vesicles and neat Ru(phen)₃ obtained at different pre-incubation times with the bacteria suspensions.

Bacterial suspension	Neat Ru(phen) ₃		Ru(phen) ₃ -loaded vesicles	
	Pre-incubation time		Pre-incubation time	
	15 min	3 h	15 min	3 h
PA ATCC	4.18	5.59	3.56	4.64
PA Iso 5	5.25	6.99	4.68	5.17
SA RN4220	5.68	6.62	1.18	1.80

Increasing the pre-incubation time from 15 min to 3 h improved the aPDT efficacy for both neat Ru(phen)₃ and Ru(phen)₃-loaded vesicles. This is evident from the higher LRFs at 3 h compared to 15 min across all samples. Longer pre-incubation may in general, enhance the cellular uptake of the Ru(phen)₃, interactions with the bacterial membrane, and intracellular localization of the photosensitizer, which is crucial for ¹O₂ generation during irradiation.¹⁵ So for optimum aPDT results, photosensitizer concentration, pre-incubation time and irradiation time are crucial parameters. Comparing aPDT effects among the bacterial strains, Ru(phen)₃-loaded vesicles were more effective against PA ATCC and PA Iso 5, and less effective against SA RN4220 even after prolonged pre-incubation.

Effect of pre-irradiating Ru(phen)₃-loaded vesicles on the aPDT efficacy

So far various aPDT parameters to improve aPDT efficacy, such as irradiation time, Ru(phen)₃ concentration in the suspension, and pre-incubation time, have been discussed in this Chapter. For further enhancements in the aPDT effects with Ru(phen)₃-loaded vesicles, the effect of pre-irradiation of the Ru(phen)₃-loaded vesicles was studied. This study aimed to determine whether pre-irradiation induces the release of Ru(phen)₃ from the vesicles into the surrounding medium, thereby increasing the availability of free Ru(phen)₃ for aPDT action. Ru(phen)₃-loaded vesicles were pre-irradiated for 30 min (15 min irradiation interval) and mixed with the bacteria suspensions, followed by 15 min incubation and 30 min irradiation (15 min ON-5 min OFF irradiation interval). So, the total irradiation time of the Ru(phen)₃-loaded vesicles was 60 min. For control, the Ru(phen)₃-loaded vesicles were tested. They were mixed with bacteria suspensions, incubated for 15 min, and then irradiated for 60 min with a 15 min ON-5 in OFF irradiation interval. For this experiment, the bacterial suspensions of PA ATCC, PA Iso 5, and SA RN4220 were used. After treatment, 10 µL aliquots of each sample were plated on agar, and Figure 9 shows the resulting log₁₀ CFU/mL.

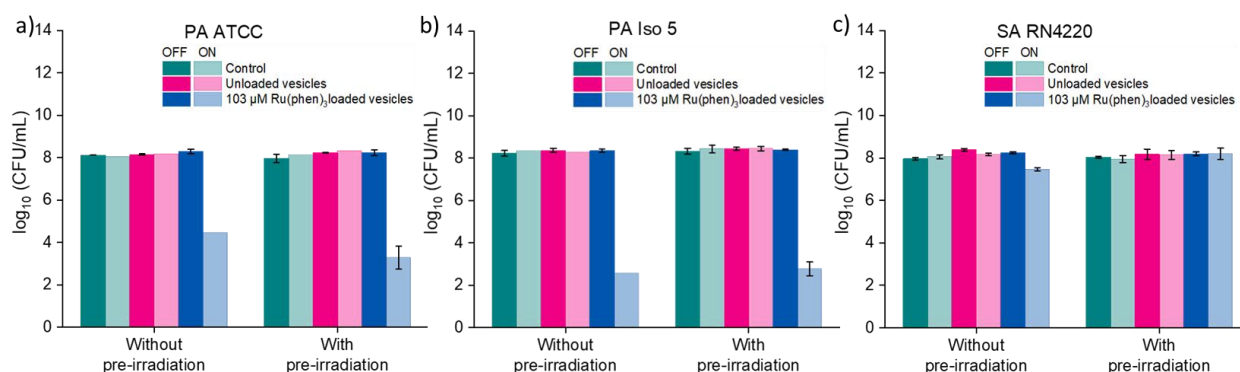


Figure 9: Bar plots of log₁₀ CFU/mL for a) PA ATCC, b) PA Iso 5, and c) SA RN4220 in 0.9% saline treated with 103 µM Ru(phen)₃-loaded vesicles without and with pre-irradiating the Ru(phen)₃-loaded vesicles. The samples were either incubated in the dark (OFF) or irradiated (ON) at $\lambda = 455$ nm. The error bars correspond to the standard deviation of technical replicates ($n = 3$).

For each bacterial suspension, no change in CFUs was observed for bacterial controls and unloaded vesicles in any experimental condition. For Ru(phen)₃-loaded vesicles, a change in CFUs was observed after irradiation. The resulting log reductions in bacterial count after the studied experimental conditions are compiled in Table 7.

Table 7: LRF for the different bacteria suspensions treated with pre-irradiated 103 μM Ru(phen)₃-loaded vesicles and without pre-irradiation of loaded vesicles before mixing with the bacteria suspensions.

Bacterial suspension	Without pre-irradiation	With pre-irradiation
PA ATCC	3.67	4.67
PA Iso 5	5.65	5.54
SA RN4220	0.49	0.27

For PA ATCC, the pre-irradiation of Ru(phen)₃-loaded vesicles resulted in a 1 log improvement in the aPDT efficacy, whereas for PA Iso 5 and SA RN4220, no improvement in aPDT effects was observed. These results indicate that pre-irradiation did not result in leaching of the Ru(phen)₃ from the encapsulated vesicles.

7.2.3 aPDT effect of Ru(phen)₃ against pathogenic *E. coli*

The aPDT effects of Ru(phen)₃ were also tested against non-pathogenic MACH-1 and pathogenic *E. coli* NCTC. The bacteria suspensions were prepared in 0.9% saline (10⁶ CFU/mL) and incubated with neat Ru(phen)₃ (final Ru(phen)₃ concentration 50 μM) for 15 min. The samples were either irradiated for different intervals or kept in the dark. The samples were serially diluted, and 5 μL of the aliquots were plated on agar for CFU counting. Figure 10 shows the resulting CFU/mL plots of both bacteria suspensions treated with neat Ru(phen)₃.

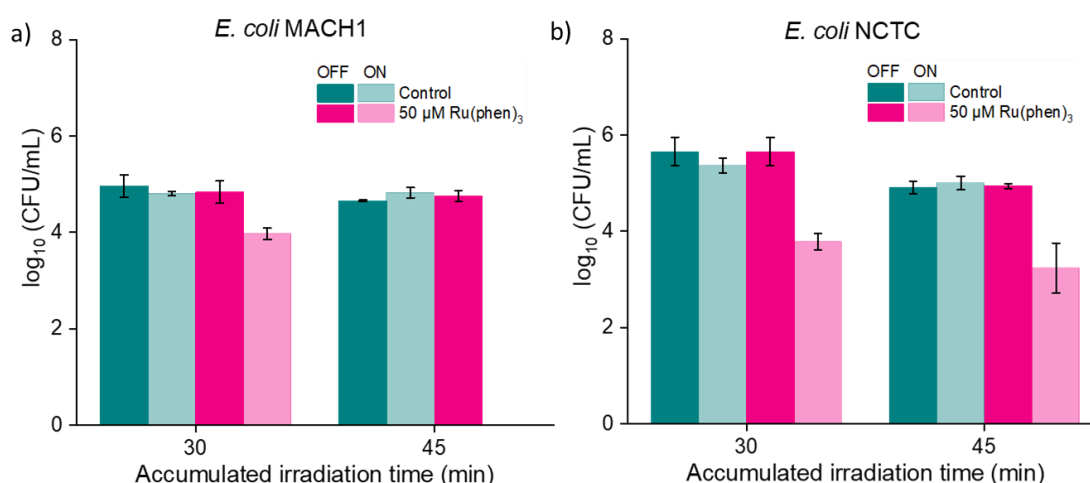


Figure 10: Bar plots of log₁₀ CFU/mL for a) *E. coli* MACH1, and b) *E. coli* NCTC in 0.9% saline treated with 50 μM Ru(phen)₃. The samples were either incubated in the dark (OFF) or irradiated (ON) at $\lambda = 455$ nm. The error bars correspond to the standard deviation of technical replicates ($n = 3$).

It was observed that there was no change in the CFUs for the bacterial controls after irradiation. In the presence of Ru(phen)₃, the aPDT effect was observed in case of both bacterial suspensions. For MACH1, no countable colonies were observed after 45 min irradiation. The LRF obtained after considering the detection limits of the colony counting for both bacteria suspensions after irradiation is given in Table 8.

Table 8: LRF for the *E. coli* bacteria suspensions treated with neat 50 μ M Ru(phen)₃ obtained after different irradiation times.

Bacterial suspension	Irradiation time (min)	
	30	45
<i>E. coli</i> MACH1	0.98	2.84
<i>E. coli</i> NCTC	1.88	1.68

The bactericidal effect was observed for *E. coli* MACH1 after 45 min irradiation, with an LRF of 2.84. Whereas *E. coli* NCTC showed moderate susceptibility to aPDT, with LRF values of 1.88 (30 min) and 1.68 (45 min), suggesting limited additional effect beyond 30 min irradiation. These findings demonstrate that neat Ru(phen)₃ exhibits strain-dependent aPDT efficacy. Optimization of aPDT parameters, including photosensitizer concentration, pre-incubation times, and oxygen availability, may be necessary to enhance efficacy against more resistant or pathogenic *E. coli* strains like NCTC.

7.3 Conclusion

This Chapter demonstrated the aPDT potential of both neat Ru(phen)₃ and Ru(phen)₃-loaded vesicles against clinically relevant *P. aeruginosa* and *S. aureus* isolates. Neat Ru(phen)₃ exhibited bactericidal activity against *P. aeruginosa* CF isolates with varying antibiotic resistance profiles, achieving >6 log reductions in four isolates after 30 min irradiation. *P. aeruginosa* is known for its high intrinsic and acquired antibiotic resistance, including efflux pumps, β -lactamases, and biofilm formation. Achieving >6 log reductions with Ru(phen)₃ alone (without adjuvants or delivery vehicles) suggests a mechanism independent of traditional resistance pathways, highlighting a key advantage of aPDT. In comparison, Ru(phen)₃-loaded vesicles achieved 99% bacterial eradication, with two isolates showing >4 log reductions due to different resistance profiles against antibiotics. Longer irradiation time was required to inactivate the most resistant strain, attributed to stronger antioxidant defenses, physical shielding effects, and inefficient photosensitizer localization, which collectively reduce the immediate aPDT effects. For *S. aureus* CF isolates, neat Ru(phen)₃ against *S. aureus* CF isolates also demonstrated significant photoactivation, achieving >5 log reductions after 45 min irradiation. The efficacy of Ru(phen)₃-loaded vesicles against *S. aureus* was comparatively lower, but improved with extended irradiation (60 min) and a higher Ru(phen)₃

concentration (135 μ M) due to enhanced photosensitizer availability and prolonged oxidative stress on bacterial cells. The pre-incubation of bacteria with the PS enhanced the aPDT effect for both neat Ru(phen)₃ and Ru(phen)₃-loaded vesicles, likely due to improved interaction with bacterial cells over time. Additionally, pre-irradiation of Ru(phen)₃-loaded vesicles showed improved efficacy against *P. aeruginosa* lab strain, but this effect was not consistent across other studied bacteria. Furthermore, pathogenic *E. coli* also responded to aPDT with the neat Ru(phen)₃, however, the level of susceptibility varied by strain, indicating optimization to achieve bactericidal effects. Unlike most studies that focus on laboratory strains, this work evaluates clinically relevant CF isolates. While vesicle-based delivery systems are commonly explored for controlled release, this study shows that Ru(phen)₃ encapsulation limits efficacy due to limited photosensitizer availability and reactive oxygen species diffusion, requiring higher concentrations and longer irradiation. Nevertheless, the high performance of neat Ru(phen)₃ without any adjuvants underscores its potential as a simple, potent, and resistance-independent aPDT agent.

7.4 Materials and methods

7.4.1 Materials

Samples

Tris(1,10-phenanthroline) ruthenium (II) bis(hexafluorophosphate) (Ru(phen)₃, >98%) was purchased from TCI chemicals (Germany). Phosphate buffered saline were purchased from Sigma Aldrich (Germany). Dimethyl sulfoxide (DMSO, 99% Sigma Aldrich, Germany), L(-)-Tryptophan (PanReac AppliChem, Germany), Tris 1 M buffer solution pH 8.5 (Thermo scientific, Germany), Lysogeny broth (LB, Luria/Miller: 10g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7.0 $\pm$ 0.2, Carl Roth GmbH, Germany), LB agar (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 15 g/L agar-agar, pH 7.0 $\pm$ 0.2 Carl Roth, Germany), sterile water and 0.9% saline (Versylene Fresenius, Germany), transparent flat-bottom 96 well plate (Sarstedt), and cell culture 96 black half area μ -clear well plate (Greiner Bio-One, Germany) were purchased from mentioned suppliers. Milli-Q water (for preparing LB medium and LB agar plates) was drawn from a Millipore Direct Q8 system with a resistivity of 18.2 M Ω cm (Millipore advantage A10 system, Schwalbach, with Millimark Express 40 filter, Merck, Germany).

Bacterial strains

P. aeruginosa lab strain ATCC 19660 (isolated from sepsis in Lima, Peru, and provided by ATCC) here noted as PA ATCC and *P. aeruginosa* cystic fibrosis patient strain CH2678 (isolated at Charité Berlin), CF592 (supplied by University Hospital Essen), M70565254 (University Hospital Münster), and MHH0426, MHH0927, MHH15204, MHH16530,

MHH17546, MHH17783, MHH1883, MHH2419 (Hannover Medical School), provided by Prof. Susanne Häußler, Helmholtz Centre for Infection Research, Braunschweig, Germany) were used in this Chapter.

S. aureus CF strains used in this Chapter (t11, t13, DIV-205, and DIV-228) were provided by Prof. Dr. Barbara Kahl, Institute of Medical Microbiology, University Clinics Münster, Germany. The pathogenic *E. coli* NCTC 10418 (noted as NCTC) was provided by Prof. Toby Jenkins, University of Bath, UK. The non-pathogenic *E. coli* MACH1TM-T1 (noted as MACH1) was bought from Invitrogen, California, USA.

Ru(phen)₃ solution preparation

5 mM stock solutions of Ru(phen)₃ were prepared in DMSO. The required Ru(phen)₃ concentrations were prepared by dissolving in sterile water.

7.4.2 Methods

Irradiation experiments

A homemade LED irradiation setup was used to perform the irradiation experiments (as shown in Chapter 5, Figure 27). The setup includes two panels of 225 blue LEDs (14 W, $\lambda = 450\text{-}460$ nm, HQRP LED plant grow panel lamp system, US) facing each other at adjustable distances. The panels were placed in such a way on top and bottom to have an exposure area in the center to achieve irradiation from both sides of the well plate. The irradiation intensity at the exposure area was adjusted to 2.7- 3.0 mW/cm², as measured using a PM 160 wireless handled power meter (Thorlabs, Bergkirchen, Germany).

For irradiation, the sample well plate was placed in the exposure area and was irradiated at room temperature for different irradiation times. To assess the effect of irradiation, treated samples were compared with samples not exposed to light. In the chapter, these conditions are referred to as OFF' (non-irradiated samples) and 'ON' (irradiated samples).

aPDT effect on planktonic bacteria

Overnight cultures were prepared by taking single colonies of each bacterial strain in 5.0 mL LB followed by incubating at 37°C for 18 h while shaking at 200 rpm. Then 1.0 mL of the cultures were centrifuged at 10,000 rpm for 5 min. The supernatant was discarded, and the pellet was resuspended in 0.9% NaCl. The OD at 600 nm of each bacteria solution was adjusted to 0.6 (for *P. aeruginosa* and *E. coli*) and 1.2 for *S. aureus*, corresponding to $\sim 10^8$ CFU/mL. If required, the 100x dilution was made in 0.9% NaCl to obtain a final working solution concentration of $3 \times 10^6 \pm 2 \times 10^6$ CFU/mL. Afterward, 25 μ L of both bacteria solutions were distributed in a 96 well-plate containing 25 μ L of Ru(phen)₃ solutions. Each sample condition was performed in technical triplicate (3 wells), separately for irradiated and non-irradiated samples. The samples were incubated for 15 min (or otherwise mentioned) at room

temperature and then the irradiation was performed. To evaluate the aPDT effect, 10 μ L (otherwise mentioned) of each technical replicate (well) of the irradiated and non-irradiated undiluted and serially diluted samples were spotted three times on agar plates, which results in a detection limit of the method of 67 CFU/mL (otherwise mentioned). The plates were incubated overnight at 37 °C and the next day bacteria colonies were counted for CFU/mL calculations of all sample conditions. The $\log_{10}$ CFU/mL and the LRF were then calculated. For the LRF, the respective non-irradiated bacteria suspension was taken as the control. The arithmetic mean and the standard deviation were calculated from 3 times independently performed experiments, with each experiment based on 3 technical replicates.

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Summary and Outlook

This Thesis discussed a comprehensive approach to address two of the most pressing challenges posed by AMR: the rapid detection of bacterial pathogens and the effective treatment of infections caused by MDR bacteria. In this context, this Thesis explored the improvement of established enzyme-responsive chitosan-based colorimetric sensors for developing diagnostic platforms for enhanced bacterial detection and investigated the polymeric nanocarriers loaded with Ru-based photosensitizers for the eradication of pathogenic bacteria using aPDT as new treatment modality.

On the diagnostic front, autonomously reporting chitosan-based sensing platforms were expanded for the rapid detection of *E. coli* employing β -Gus as a bacterial biomarker. The enzyme-responsive sensing moieties were incorporated both into paper-based hydrogels and electrospun nanofiber systems. The paper-based sensing platforms with smartphone-assisted RGB analysis (Chapter 3) enabled visible, low-cost, and user-friendly POC diagnostics, making it suitable for low-resource clinical settings. Upon enzymatic hydrolysis of chromogenic substrate X-Gluc incorporated into chitosan hydrogels, a distinct blue coloration was observed, serving as a reliable visual indicator of β -Gus activity. The limits of detection for visual assessment were 60 nM for neat enzyme solutions after 2 h of incubation, and $\sim 10^8$ CFU/mL for *E.coli* after 8 h. RGB analysis further elucidated the correlation between enzyme concentration, incubation time, and the colorimetric response.

To enhance sensitivity and enable scalable fabrication, this approach was extended in Chapter 4 to develop an electrospun nanofiber-based enzyme-sensing platform. The nanofibers with an average fiber diameter of 363 ± 100 nm were fabricated via electrospinning and functionalized with the fluorogenic substrate MUG for β -Gus detection. At a fixed enzyme concentration, the nanofibers demonstrated a 3.4-fold increase in the initial enzymatic hydrolysis rate compared to the hydrogel system and achieved an LOD of 4.7 nM after 60 min, compared to 16 nM for hydrogels. The enhanced sensitivity of the nanofiber platform was further validated through *in vitro* experiments using both pathogenic and non-pathogenic *E. coli* strains, underscoring its potential utility in real biological samples.

Complementing the diagnostics, this Thesis explored aPDT as a promising alternative to conventional antibiotic therapy. Ruthenium(II)-based photosensitizer (Ru(phen)₃) encapsulated within polymeric vesicles showed a greater photodynamic inactivation compared to Ru(phen)₃-loaded micelles, achieving a > 4.7 log reduction in viable bacterial counts for studied *P. aeruginosa* strains (Chapter 5). This enhanced efficacy was attributed to the higher loading capacity of Ru(phen)₃ within the vesicular structures.

In Chapter 6, the efficacy of Ru(phen)₃-loaded vesicles was further evaluated against *P. aeruginosa* biofilms and in artificial sputum medium (ASM), which mimics the CF lung environment. The biological complexity of ASM significantly influenced aPDT efficacy, with Ru(phen)₃-loaded vesicles showing reduced activity compared to the neat Ru(phen)₃ at similar overall Ru(phen)₃ concentrations. Variability in ASM stability and batch composition also contributed to the variability in aPDT outcomes. *P. aeruginosa* biofilm eradication assay demonstrated a 4.8 log reduction with neat Ru(phen)₃ and a 2 log reduction with Ru(phen)₃-loaded vesicles after 120 min of irradiation. Prolonged pre-incubation times appeared to facilitate deeper photosensitizer penetration into biofilms, thereby enhancing aPDT outcomes. These insights provide a foundation for optimizing aPDT for treating *P. aeruginosa* infections in the future, particularly in CF and other biofilm-associated infections.

The aPDT efficacy of Ru(phen)₃-loaded vesicles and neat Ru(phen)₃ was further explored in Chapter 7 against clinical isolates of *P. aeruginosa* and *S. aureus* from CF patients, as well as pathogenic *E. coli*. The neat Ru(phen)₃ exhibited bactericidal activity, with >6 log reductions in four *P. aeruginosa* isolates after 30 minutes of irradiation. Ru(phen)₃-loaded vesicles achieved up to 99% bacterial eradication, with two isolates showing >4 log reductions. For *S. aureus* CF isolates, neat Ru(phen)₃ induced >5 log reductions after 45 minutes. The efficacy of Ru(phen)₃-loaded vesicles against *S. aureus* was comparatively lower but improved with extended irradiation (60 min) and higher Ru(phen)₃ concentrations (135 μM). Pre-incubation of bacteria with Ru(phen)₃ enhanced the aPDT effects for both neat Ru(phen)₃ and Ru(phen)₃-loaded vesicles, likely due to improved photosensitizer uptake or interaction with bacterial membranes over time. Additionally, the pre-irradiation of Ru(phen)₃-loaded vesicles showed improved efficacy against *P. aeruginosa* lab strain, but this effect was not consistent across other studied bacteria. Furthermore, pathogenic *E. coli* also responded to aPDT with the neat Ru(phen)₃, however, the level of susceptibility varied by strain, indicating the need for optimization to achieve bactericidal effects.

Despite the promising results, further research is necessary to translate these platforms closer to clinical practice. For diagnostics, extending the nanofiber-based sensor design to enable multiplexed detection of diverse bacterial biomarkers would enhance their versatility and diagnostic value. On the therapeutic side, future work should focus on refining the nanocarrier systems to further improve the circulation time of photosensitizers in complex biological environments. Moreover, combining diagnostic and therapeutic modalities into a single theranostic platform could pave the way for tailored and real-time infectious disease management. In conclusion, this work lays the groundwork for the development of smart diagnostic and therapeutic strategies to address AMR. By bridging the gap between early bacteria detection and effective treatment, the approaches presented herein may in the long

run contribute to the broader effort of curbing antimicrobial resistance and improving patient outcomes in an era of diminishing antibiotic efficacy.

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