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PREPARATION AND CHARACTERIZATION OF NANOMATERIALS FOR ANTIBACTERIAL THERAPY APPLICATIONS

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Preface

This PhD thesis entitled “*Preparation and Characterization of Nanomaterials for Antibacterial Therapy Applications*” has been submitted to the Department of Biological, Chemical and Pharmaceutical Science and Technologies (STEBICEF) at the University of Palermo to obtain the PhD degree in "Technologies and Sciences for Human Health" on 26th February 2025; and to the Graduate School of Health and Medical Sciences at the University of Copenhagen, on 26th February 2025.

The present project was conducted within a double PhD degree program between the University of Palermo (leading/home institution) in Italy and the University of Copenhagen (secondary/host institution) in Denmark.

For the periods June 2022, and from July 2023 to December 2023, research was conducted at the host university, within the Drug Delivery and Biophysics of Biopharmaceuticals group at the Department of Pharmacy, University of Copenhagen, under the supervision of Professor Vito Foderà.

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- Francesca Terracina, Sergio Sciré, **Giorgia Puleo**, Valentina Catania, Attilio Ignazio Lo Monte, Domenico Schillaci, Mariano Licciardi. *Polybutylene succinate-based electrospun scaffolds improving skin permeation of the antibacterial ciprofloxacin*. Submitted to Journal of Drug Delivery Science and Technology.
- **Giorgia Puleo**, Vito Foderà, Valeria Vetri et al. *Enhanced Oxidative Photodegradation of DNA and Lipid Membranes via Composite Nitrogen-Doped Titanium Dioxide with Gold Nanorods*. Manuscript in preparation.
- **Giorgia Puleo**, Valeria Vetri, Vito Foderà, et al. *Lignin-based Nanofibers by Green Electrospinning for Tuneable Biomedical Applications*. Manuscript in preparation.

The content of this thesis is outlined as follows:

- In **Chapter 1**, a general introduction to the state of the art of commonly employed therapeutic approaches is provided, highlighting their limitations and potential novel therapeutic applications. The focus is on nanostructured materials for antibacterial photodynamic therapy and nanofibers developed by electrospinning.
- **Chapter 2** outlines the fundamental principles of the experimental methodologies employed in this research. Particular emphasis is placed on the role of advanced production and characterization techniques in enabling the design of novel biomaterials and the precise assessment of their physicochemical properties.

- In **Chapter 3**, the photocatalytic properties of nitrogen-doped titanium dioxide nanostructures combined with gold nanorods are explored under UV-visible light illumination. Their combination enhances the photocatalytic effect, leading to the degradation of target biomolecules essential for bacterial viability. Absorption and fluorescence spectroscopy were employed, utilizing fluorescent probes such as C-11 BODIPY and Laurdan, which provided valuable insights into molecular details underling oxidative effects on lipid membranes.
- In **Chapter 4**, the sustainable green production of lignin-based nanofibers is presented by waterborne electrospinning, aiming at valorising lignin, a material often discarded due to its heterogeneity and challenging processability, and exploiting its significant biotechnological properties. The developed nanofibers exhibited promising tuneable features, such as autofluorescence, mechanical strength, and antioxidant activity, all regulated by the packing of lignin molecule within the fibers. As a proof of concept, this system was evaluated as a controlled drug delivery platform for a bacitracin as antibiotic molecule
- In **Chapter 5**, the production of polymeric microparticles via electrospraying, based on the biodegradable and biocompatible polymer is described and their use as drug delivery system was assessed. These microparticles were loaded with Ciprofloxacin, used as a model molecule for a poorly water-soluble antibiotic, demonstrating their capability for sustained drug release, enhanced permeability, and antibacterial and antibiofilm efficacy.
- In **Chapter 6**, a summary of the conclusions and potential future research perspectives is provided.

It is hereby disclosed that during the writing of the thesis, to improve readability and clarity and to check grammatical errors, OpenAi's ChatGPT-4 model was used. In any case, after use, any output was then reviewed and modified, therefore the accuracy and originality of the work is guaranteed, and full responsibility is assumed for the contents reported here.

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Abstract

The increasing development of antibiotic resistance poses a major challenge to be addressed to ensure global health, as it undermines the efficacy of conventional antibacterial therapies. These treatments, often reliant on broad-spectrum antibiotics, are limited by reduced efficacy, off-target effects, and the emergence of resistant microbial strains. This crisis necessitates the urgent development of innovative therapeutic strategies that overcome the limitations of current approaches while maintaining precision and efficacy.

Among the promising solutions, photocatalytic biomaterials offer unique opportunities to address these challenges. The size and structure of biomaterials are crucial factors in their interaction with biological systems, influencing drug delivery, cellular uptake, and antibacterial activity. Nanostructured materials, in particular, provide high surface-area-to-volume ratios, tuneable physicochemical properties, and the ability to precisely target pathogenic sites. These characteristics make them ideal candidates for overcoming the barriers faced by conventional therapies.

This thesis investigates the design, synthesis, and application of innovative nanostructured biomaterials tailored for antibacterial applications. Key contributions include the development of nitrogen-doped titanium dioxide and gold nanorods composites for photodynamic therapy. These materials demonstrated efficacy under visible light irradiation, inducing oxidative damage in biomolecular targets such as DNA and lipid membranes, evaluated through advanced spectroscopic methods.

Sustainability in biomaterial design is exemplified through the innovative use of lignin, a renewable and underutilized biopolymer, for nanofiber production. By employing green synthesis methods, lignin was transformed into functional nanofibers with intrinsic autofluorescence, antioxidant activity, and mechanical properties. These nanofibers demonstrated potential as carriers for therapeutic molecules, such as bacitracin, offering an environmentally friendly alternative for biomedical applications.

The study also explores polymeric microparticles produced through electrospraying, utilizing biodegradable polybutylene succinate (PBS) as a polymeric carrier for the controlled delivery of ciprofloxacin. This system addresses the challenges of poorly water-soluble antibiotics, demonstrating sustained release, enhanced drug permeability, and strong antibacterial and antibiofilm properties.

By addressing the limitations of conventional therapies and introducing sustainable, multidimensional biomaterial solutions, this work provides a significant contribution to the development of next-generation therapeutic platforms. The findings emphasize the critical importance of biomaterial design and functionality in creating targeted, effective, and environmentally conscious solutions for pressing global health challenges.

Resumé

Fremkomsten af antibiotikaresistens udgør en betydelig global sundhedsudfordring, der underminerer effektiviteten af konventionelle antibakterielle behandlinger. Disse behandlinger, der ofte er afhængige af bredspektrede antibiotika, er begrænsede af reduceret effektivitet, off-target-effekter og fremkomsten af resistente mikrobielle stammer. Denne krise nødvendiggør en hurtig udvikling af innovative terapeutiske strategier, der overvinder begrænsningerne ved nuværende metoder og samtidig opretholder præcision og effektivitet.

Blandt de lovende løsninger tilbyder avancerede biomaterialer unikke muligheder for at imødegå disse udfordringer. Biomaterialernes størrelse og struktur spiller en afgørende rolle i deres interaktion med biologiske systemer, hvilket påvirker lægemiddellevering, cellulært optag og antibakteriel aktivitet. Særligt nanostrukturerede materialer giver høje overflade-til-volumen-forhold, justerbare fysikokemiske egenskaber og evnen til præcist at målrette patogene steder. Disse egenskaber gør dem til ideelle kandidater til at overvinde barrierer ved konventionelle terapier.

Denne afhandling undersøger design, syntese og anvendelse af innovative biomaterialer skræddersyet til antibakterielle formål. Centrale bidrag inkluderer udviklingen af kvælstofdoteret titaniumdioxid- og guldnanorods-kompositter til fotodynamisk terapi. Disse materialer viste effektivitet under bestråling med synligt lys, hvilket inducerede oxidativ skade på biomolekylære mål som DNA og lipidmembraner, vurderet gennem avancerede spektroskopiske metoder.

Bæredygtighed i biomaterialedesign eksemplificeres gennem den innovative brug af lignin, en fornybar og underudnyttet biopolymer, til produktion af nanofibre. Ved anvendelse af grønne syntesemetoder blev lignin omdannet til funktionelle nanofibre med iboende autofluorescens, antioxidantaktivitet og mekaniske egenskaber. Disse nanofibre viste potentiale som bærere for terapeutiske molekyler, såsom bacitracin, og tilbyder et miljøvenligt alternativ til biomedicinske anvendelser.

Studiet undersøger også polymere mikropartikler fremstillet gennem elektrospay, hvor biologisk nedbrydeligt polybutylensuccinat (PBS) anvendes som matrix til levering af ciprofloxacin. Dette system adresserer udfordringerne ved antibiotika med lav vandopløselighed og demonstrerer forlænget frigivelse, forbedret lægemiddelpermeabilitet samt stærke antibakterielle og antibiofilm-egenskaber.

Ved at adressere begrænsningerne ved konventionelle behandlinger og introducere bæredygtige, multidimensionelle biomaterialeløsninger bidrager dette arbejde væsentligt til udviklingen af næste generations terapeutiske platforme. Resultaterne understreger den kritiske betydning af biomateriale design og funktionalitet i skabelsen af målrettede, effektive og miljøbevidste løsninger til presserende globale sundhedsudfordringer.

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Acronyms

aPDT	Antibacterial Photodynamic Therapy
ATR-FTIR	Attenuated Total Reflectance - Fourier Transform InfraRed
AuNRs	Gold Nanorods
Bac	Bacitracin
BODIPY	C-11 BODIPY ^{581/591}
CB	Conduction Band
CLSM	Confocal Laser Scanning Microscopy
CPX	Ciprofloxacin
CTAB	Cetyltrimethylammonium Bromide
CT-DNA	Calf Thymus DNA
DDS	Drug Delivery System
DLS	Dynamic Light Scattering
DMA	Dynamic Mechanical Analysis
DPPH	2,2-Diphenyl-1-picrylhydrazyl
EDX	Energy Dispersive X-ray
FLIM	Fluorescence Lifetime Imaging Microscopy
GP	Generalized Polarization
HPLC	High-Performance Liquid Chromatography
LSPR	Localized Surface Plasmon Resonance
MO	Methyl Orange
MPs	Microparticles
N-TiO₂	Nitrogen-Doped Titanium Dioxide

Acronyms.

NIR	Near-Infrared
PAPC	1-Palmitoyl-2-arachidonoyl-sn-phosphatidylcholine
PBS	Polybutylene succinate
PEO	Polyethylene Oxide
p-MPs	Polymeric Microparticles
POPC	1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
POPG	1-Palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol)
RH	Relative Humidity
ROS	Reactive Oxygen Species
SEM	Scanning Electron Microscopy
STEM	Scanning Transmission Electron Microscopy
SUVs	Small Unilamellar Vesicles
TEM	Transmission Electron Microscopy
UV-Vis	Ultraviolet-Visible
VB	Valence Band

Chapter 1

Introduction

This chapter explores the state-of-the-art challenges related to bacterial infections in the context of rising antimicrobial resistance, emphasizing the limitations of traditional antibiotic therapies and the urgent need for innovative solutions. It explores current approaches to combat multidrug-resistant strains, with a focus on different advanced strategies, from photodynamic therapy, to electrospun nanofibers, and to polymeric microparticles.

1.1 Current Challenges in Antibacterial Therapies

Antibacterial resistance is a significant worldwide health issue, characterized by the declining efficacy of antibiotics in combating bacterial infections because of the rise of multidrug-resistant strains (MDR) strains [1,2]. This phenomenon, driven by the overuse and misuse of antibiotics and the adaptive capabilities of bacteria, threatens to undermine decades of progress in modern medicine. The World Health Organization has identified antimicrobial resistance as one of the ten most significant threats to global public health, emphasizing the critical necessity for novel treatment strategies to tackle this growing crisis [3,4].

Traditional treatments for bacterial infections primarily rely on antibiotics targeting key cellular processes, including the synthesis of the cell wall (like beta-lactams), the production of proteins (such as macrolides), and the replication of DNA (for example, fluoroquinolones). While effective against susceptible pathogens, these treatments often fail against resistant strains due to mechanisms such as efflux pumps, enzymatic degradation, and genetic mutations [5].

Biofilms are highly structured multicellular communities that adhere to surfaces, such as prosthetic devices and medical implants, creating a protective environment for bacterial survival [6]. These structures are embedded in a dense extracellular polymeric matrix that not only limits antibiotic penetration but also capable of deactivating antimicrobial agents. Within this matrix, bacteria exchange genetic material through horizontal gene transfer, further enhancing resistance. Additionally, biofilms contain metabolically heterogeneous bacterial populations, including "persister" cells, which enter a

dormant state. These cells evade antibiotics that target active cellular processes, contributing to chronic infections and treatment failure [7].

Most antibiotics used in modern medicine were identified during the period known as the "golden age" of antibiotic discovery, which took place between the 1940s and 1960s. However, the decades following this period have witnessed a pronounced decline in the development of novel antibiotics to several reasons [8,9]. Most of the antibiotics introduced in recent years are modifications of existing drug classes, primarily designed to improve specific pharmacokinetic and pharmacodynamic characteristics [10]. While these modifications have extended the utility of some antibiotics, they have not addressed the fundamental issue of resistance proliferation, leaving significant gaps in the current therapeutic arsenal.

The crisis of antibacterial resistance underscores the necessity of developing innovative therapeutic strategies that go beyond the limitations of current treatments. The upcoming discussion will focus on the major challenges faced by contemporary antibacterial therapies, including their inherent limitations, and explore potential approaches to address these challenges. These advancements are essential not only for enhancing treatment efficacy as well as enhancing patients' health outcomes and their overall quality of life.

Traditional approaches to treating bacterial infections rely on the use of antibiotics, which can either suppress the growth of bacteria or eliminate them, based on the specific characteristics of Gram-positive and Gram-negative bacteria. This approach, however, is inherently constrained by several factors, including the limited availability of antibiotics, their spectrum of activity, and the increasing prevalence of antibiotic resistance [11]. As resistance mechanisms evolve, bacteria are increasingly able to withstand the effects of many commonly used antibiotics, reducing the efficacy of traditional treatments. Consequently, this has led to a growing challenge in clinical settings, making it essential to develop innovative therapeutic approaches to tackle infections that no longer respond to conventional antibiotic treatments.

To address this increasingly urgent issue, researchers have focused on both exploring novel synthetic solutions and identifying alternative therapeutic approaches. In terms of novel solutions, high-throughput screening of large molecular libraries has been widely employed to discover new drug candidates [12,13]. Alternative strategies have aimed at improving the selectivity and delivery of existing drugs, as well as counteracting bacterial resistance to restore the effectiveness of established antimicrobial agents [14].

A particularly promising research area in this field is nanomedicine, with the goal of improving the accuracy, effectiveness, and bioavailability of antimicrobial compounds [15]. The size of materials is significant in influencing their biological interactions and therapeutic potential. As the scale decreases

from the micrometer to the nanometer range, materials exhibit distinctive physicochemical properties, including tuneable size, a high ratio of surface area to volume, and the ability to be functionalized, which are pivotal for overcoming biological barriers. Nanostructured materials, including nanoparticles, nanofibers, and larger assemblies that retain nanoscale properties, offer remarkable opportunities for therapeutic innovation. These materials can bypass bacterial defences, disrupt biofilms, and enable targeted delivery to infection sites, reducing systemic toxicity [16,17]. Various types of nanostructured materials are presently under investigation to enhance the effectiveness of bacterial infection treatments.. These systems enhance drug delivery by optimizing therapeutic efficacy and enabling more precise targeting of infections [18,19].

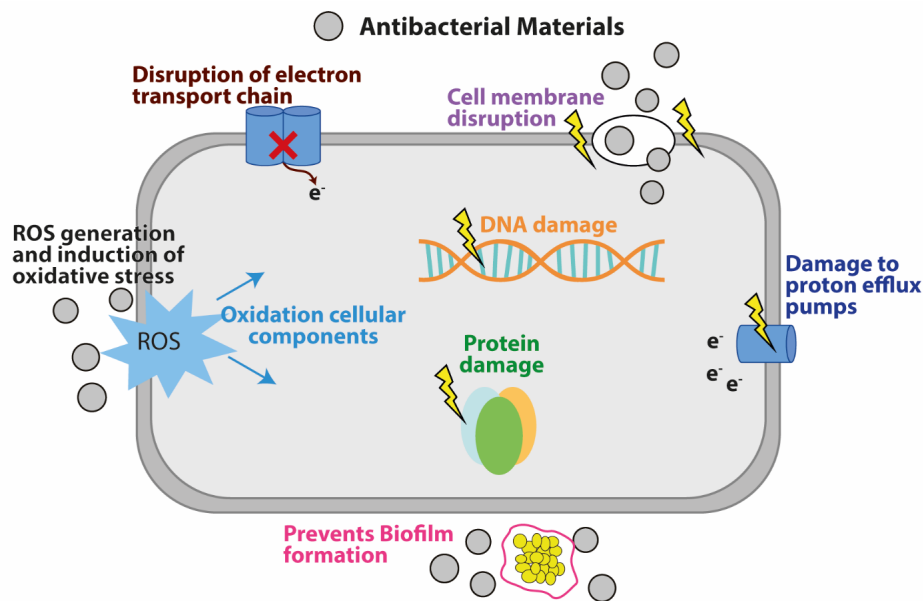


Figure 1: Antibacterial materials act through various mechanisms, including cell membrane disruption, DNA damage, protein oxidation, inhibition of the electron transport chain, impairment of proton efflux pumps, induction of oxidative stress via ROS generation, and prevention of biofilm formation. Reproduced with adaptations in accordance with copyright use rules from [15].

Antibacterial materials can carry out their effects through several distinct mechanisms, as summarized in Figure 1. These materials can directly interact with bacterial membranes, modifying membrane permeability, and also disrupting both active and passive ion transport by interfering with electron transport chains and impairing efflux pumps. Moreover, thanks to their size and tuneable characteristics some materials showed to be able to prevent biofilm formation. For instance, positively charged lipid-based particles are capable of penetrating the biofilm matrix and delivering the drugs internally [20]. One of the primary mechanisms through which these agents display antibacterial activity is by causing oxidative stress, resulting from the production of Reactive Oxygen Species (ROS), that diffuse within the cell and oxidize its components. Notably, metallic-based antibacterial materials are well recognized for their ability to generate ROS, with the specific type of ROS produced

varying according to the metal involved [21,22].

In addition to these approaches, light-based therapies including photothermal therapy (PTT) and photodynamic therapy (PDT), have also gained attention as promising alternatives for treating bacterial infections resulting from antibiotic-resistant strains. PDT utilizes photosensitizing agents that, when activated by specific wavelengths of light, produce ROS capable of damaging and killing bacteria [23–25]. This approach can effectively target biofilms and resistant bacteria, offering a non-antibiotic method to tackle infections. Moreover, light therapy has the advantage of minimal systemic side effects and can be precisely controlled, making it a valuable adjunct to traditional treatments.

PTT, on the other hand, exploits photothermal agents, such as metal nanoparticles, that upon irradiation can generate localized hyperthermia. The rise in temperature at the local level damages bacterial cell membranes, causes protein denaturation and eventually results in the death of bacterial cells [26]. PTT has shown particular promise for treating surface-level infections and biofilm-associated bacterial colonies, as the elevated temperatures can penetrate tissues and provide effective treatment even in deeper or more complex infection sites [27]. Gold NPs combined with polyphenols showed efficacy against multidrug-resistant bacteria and biofilm-infected wounds by photothermal therapy under NIR irradiation [28]. Both PDT and PTT offer significant advantages, including their ability to target localized areas with high precision, reducing the risk of collateral damage to nearby tissues that do not require treatment. Furthermore, these treatments may be combined with other antimicrobial agents to potentiate their effects, or as standalone treatments where antibiotics may no longer be effective [29]. Lastly, in the landscape of treatment options, smart biomaterials have emerged as a key component, complementing various strategies for tackling bacterial infections [30]. These advanced materials, which include hydrogels, electrospun nanofibers, 3D-printed structures, and polymeric microparticles, are usually composed of natural or synthetic polymers and can be tailored to react to various external and internal triggers, including pH levels, temperature changes, light exposure, or enzymatic processes. Their ability to adapt to microenvironmental changes enhances therapeutic efficacy by enabling precise and localized interventions [31–34]. Hydrogels, such as those derived from chitosan or alginate, can release antibiotics in response to acidic environments typical of infected tissues, ensuring localized and effective treatment [35,36]. Similarly, electrospun nanofibers can incorporate silver nanoparticles or gentamicin serving as both a physical barrier to bacterial colonization and a source of sustained antimicrobial agent release, proving effective in preventing biofilm formation and promoting tissue healing, as demonstrated in wound and surgical site applications [37,38]. Polymeric microparticles also offer several advantages in the delivery of antimicrobial agents. These microscale systems, often fabricated from biocompatible materials including poly(lactic-co-glycolic acid) (PLGA), polyvinylpyrrolidone (PVP), polylactic acid (PLA), but also alternatively natural derived polymers

like cellulose or gelatine, are specifically tailored to encapsulate therapeutic substances and ensure their controlled and sustained release. For example, polymeric microparticles have been successfully used to deliver daptomycin and vancomycin, ensuring extended therapeutic effects at the infection site and reducing systemic side effects [39]. Additionally, their larger size compared to nanoparticles reduces rapid clearance by the body, making them particularly useful for long-term applications.

3D-printed scaffolds made from biocompatible polymers like polycaprolactone, embedded with antibiotics or antimicrobial peptides, have been successfully employed in orthopaedic implants, simultaneously addressing bacterial infections and bone regeneration needs [40].

In the context of this thesis, we will focus on innovative approaches, placing particular emphasis on photodynamic therapy, electrospun nanofibers, and polymeric microparticles. These systems provide the opportunity to explore different techniques of production and characterization methods, which will be thoroughly examined in the following sections.

1.2 Antibacterial Photodynamic Therapy

By exploiting the on-demand activation of antibacterial treatment and the ROS generated to target bacterial infections, PDT has emerged as one of the most promising antibacterial therapeutic strategies. Initially studied and applied in cancer therapy, where it is already a clinical reality in many countries [41], it has more recently been adapted for antibacterial applications (aPDT), particularly for targeting multidrug-resistant bacteria, due to its effectiveness [42]. This therapy relies on molecules known as photosensitizers, which, upon exposure to specific wavelengths of light, undergo electronic rearrangement. This process leads to the generation of ROS via two primary pathways: Type I and Type II reactions [43]. In Type I reactions are generated free radicals like superoxide anions and hydroxyl radicals, while Type II reactions produce singlet oxygen. These ROS initiate a cascade of reactions that cause significant damage to nearby targets, including tumour cells in cancer therapy or bacteria and biofilms in aPDT. In Figure 2 the mechanisms that occur are described in a modified typical Jablonski diagram.

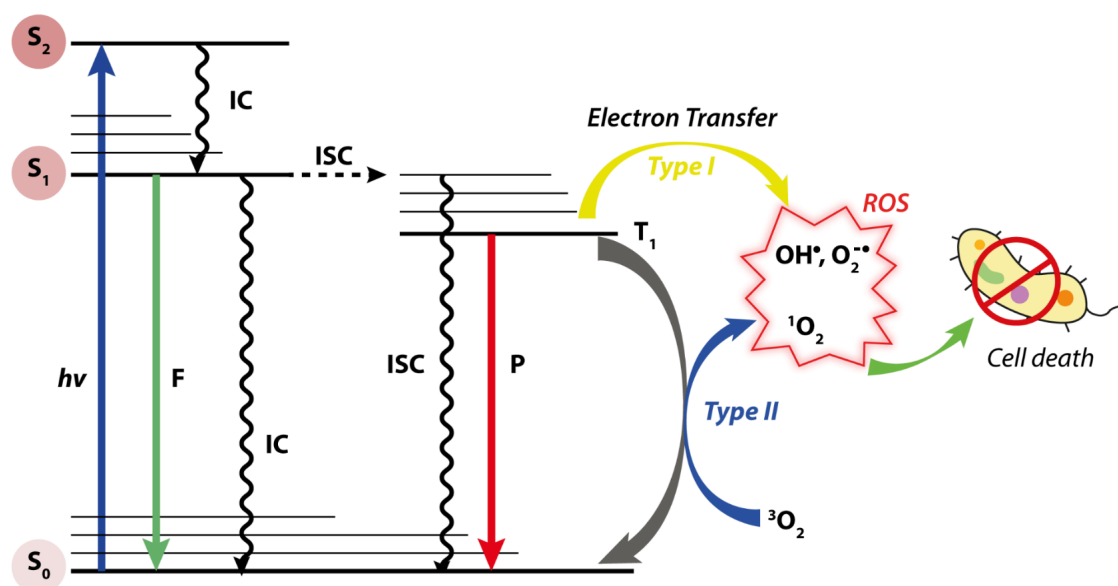


Figure 2: **Jablonski diagram illustrating the photodynamic therapy (PDT) mechanism.** Upon light absorption ($h\nu$), a transition of the photosensitizer from the minimum energy, ground state (S_0) to one of the excited singlet states (S_1 or S_2). Non-radiative relaxation occurs via internal conversion (IC), while fluorescence (F) is emitted during relaxation from S_1 to S_0 . Intersystem crossing (ISC) enables transition to the triplet state (T_1), initiating two pathways: Type I, generating ROS such as $\text{OH}^\bullet$ and $\text{O}_2^{\bullet\bullet}$, and Type II, producing singlet oxygen ($^1\text{O}_2$). Both pathways result in ROS production leading to oxidative damage and cell death [44]. Reproduced and adapted with permission in accordance with copyright use rules from [45].

When illuminated with light at the proper wavelength, the photosensitizer absorbs a photon, undergoing a transition that causes it to move from its lower energy, ground state (S_0) to a higher energy state, the excited singlet (S_2). It can then dissipate energy through different processes, such as fluorescence (F) or heat via internal conversion (IC), thereby returning to the ground state [46]. Alternatively, intersystem crossing (ISC) can occur, leading to the conversion to a longer-living excited triplet state (T_1). From this state, the photosensitizer may return to the ground state via phosphorescence emission or trigger the production of ROS through the aforementioned Type I and Type II reactions [47–49]. Type I reactions, in particular, involve the transfer of electrons to surrounding molecules. When this transfer involves the molecular oxygen (O_2), often in aqueous environment, this generates superoxide anions ($\text{O}_2^{\bullet-}$). These superoxide radicals can then react further, undergoing dismutation to form hydrogen peroxide (H_2O_2). When these reactions occur with the participation of transition metals, for example, iron, Fenton or Fenton-like reactions can occur, producing highly reactive hydroxyl radicals ($\text{HO}^\bullet$) capable of damaging cellular components through oxidation. On the other hand, Type II reactions involve the transfer of the energy directly to surrounding oxygen, leading to the formation of singlet oxygen ($^1\text{O}_2$), which is highly reactive. Both mechanisms, through their production of ROS, contribute to cellular damage. The efficacy of PDT is attributed to the extensive reactivity of these molecules, as they can initiate oxidative chain reactions

with a variety of surrounding compounds, including proteins, lipids, and nucleic acids [50,51]. This non-selective nature distinguishes PDT from traditional antimicrobial approaches [42]. In general, resistance development is more common in treatments that target a specific structure or pathway, such as antibiotics or antiseptics, where pathogens can evolve resistance through specific mutations, efflux pump expression, or the upregulation of protective enzymes [52,53]. The multi-target approach is thought to minimize the likelihood of pathogens developing resistance, as evidenced by studies suggesting that resistance to aPDT is highly unlikely [42,54]. Moreover, another crucial advantage of this therapeutic strategy is that both cancer cells and bacterial cells show a marked susceptibility compared to healthy human tissues. This differential vulnerability is likely due to the higher metabolic activity and oxidative stress already present in cancerous and microbial cells. Furthermore, the specificity of aPDT can be further improved by precisely targeting photosensitizers using advanced drug delivery systems (DDS). These systems allow for the targeted accumulation of the photosensitizer in tumour tissues or infected areas, minimizing damage to surrounding healthy tissues. And since the illumination required to activate these photosensitizers can be localized, this enables another layer of selectivity by enabling on-demand treatment precisely at the site of the tumour or inflammation. This spatial control significantly improves the safety and effectiveness of photodynamic therapy, making it a promising approach for treating both infections and cancer without widespread collateral damage. The effectiveness of PDT is primarily driven by the photosensitizer molecule. In the literature, a wide range of molecules are identified as potential photosensitizers, capable of triggering these reactions by absorbing light and transitioning to an excited state [55]. The most well-known and extensively studied photosensitizers are typically categorized into three main types: porphyrins, phthalocyanines, and chlorins [56]. Porphyrins are the most commonly used due to their efficient photosensitization properties, while phthalocyanines and chlorins offer advantages such as deeper tissue penetration and reduced side effects. However, these molecules often exhibit limitations, including poor solubility in aqueous solutions and reduced stability under prolonged light exposure, which can hinder their clinical application [57–59].

In addition to organic molecules, inorganic photosensitizers, such as semiconductor oxides like titanium dioxide (TiO_2) and zinc oxide (ZnO), metal nanoparticles, and metal complexes, are emerging as promising alternatives [60–62]. These materials absorb UV or visible light and generate ROS through slightly different mechanisms compared to organic photosensitizers. Their properties can be tailored to better suit various therapeutic applications, offering benefits like enhanced stability, non-toxicity, and efficient light absorption. Moreover, specific synthetic strategies can be employed to create inorganic photosensitizers in the form of nanomaterials, harnessing the distinctive properties of materials in the nanoscale range to improve treatment outcomes. The high surface area and increased

reactivity of these nanomaterials enable better interaction with target tissues, thus enhancing treatment efficacy while minimizing side effects.

1.3 Tuneable Biomaterial for Antibacterial Applications

In the context of advanced and innovative therapies, it is essential to acknowledge that the therapeutic agent needs an effective delivery system capable of targeting specific sites and enhancing absorption to fulfill its intended function. Among the most adaptable delivery systems, biomaterials stand out as suitable options for various localized applications and surgical procedures [63]. Although the term "biomaterials" encompasses a wide range of concepts and is challenging to define precisely, it can generally refer to structures, often based on synthetic or natural polymers, integrated with diverse active agents or drugs. These materials interact with biological systems to treat, heal, or replace physiological functions. The design and development of biomaterials are pivotal for their intended specific application. Such meticulous design allows for selective optimization, conferring tuneable properties that can improve the pharmacokinetic profile of the incorporated drug [64].

Examples of tuneable properties include mechanical properties, porosity, antioxidant activity, surface charge, biocompatibility, and degradation rate. The regulation of these properties not only improves drug release profiles but also the interaction with target tissues and the compatibility with the biological environment. The combined effect of these improvements aims to ensure a more effective treatment, with minimal side effects [65].

For instance, surface charge modification with cationic polymers like chitosan or polyethyleneimine disrupts bacterial membranes by electrostatic interaction, a mechanism demonstrated in studies on chitosan-coated implants and PEI-based hydrogels [66,67].

Antioxidant properties are increasingly recognized as crucial for biomaterials used in inflammation-prone environments. For example, hydrogels based on tannic acid and polyethylene glycol have demonstrated high antioxidant capacity as well as antibacterial activity against bacterial strains such as *S. aureus* and *E. coli* [68,69]. Similarly, polymer matrices incorporating nanoparticles of other metal oxides such as cerium (CeO_2), showed dual functionality by acting as scavengers of ROS and as antibacterial agents. Interestingly, as the nanoparticles interact with the polymer functional groups, stable complexes are formed, which significantly improve the nanoparticles' biocompatibility and overall performance [70].

In the field of biomaterials design, mechanical properties are essential in structural applications such as bone or cartilage regeneration, as well as wound healing applications. In these contexts, factors such as strength and elasticity are key parameters. For instance materials like PLGA or hydroxyapatite composites can mimic native tissue properties, maintain stability and proper performance in

physiological settings, and allow for cell adhesion and growth [71,72]. Dynamic features like stiffness gradients and viscoelasticity enhance tissue integration. These properties are also crucial for enabling biomaterials to conform to body shapes while maintaining structural integrity under stress, reducing fracture risks [73,74].

Biocompatibility, a key property in biomaterial design, can be achieved through surface functionalization with bioactive molecules, green synthesis free of toxic agents, or using natural products as starting materials. For instance, functionalizing titanium with bioactive peptides improves osseointegration [75], green synthesis using plant extracts enhances nanoparticle safety [76,77] and natural polymers like chitosan offer intrinsic biocompatibility [67,78].

Combined all together, these properties, carefully engineered through advanced biomaterial science, enable the development of multifunctional systems that address clinical challenges effectively. The integration of tuneable antibacterial, antioxidant, mechanical, and biocompatibility features exemplifies the progress in this field and its transformative impact on medical applications.

Among the various existing biomaterials, this thesis focuses on electrospun scaffold materials. In the following section, their most interesting properties will be discussed in the context of tissue engineering, wound healing, and antibacterial treatments.

1.4 Electrospun nanofibers with Functional Properties

The remarkable versatility of electrospun nanofibers, which can assemble into interconnected networks to form scaffolds, has positioned them as highly valuable multifunctional biomaterials for various biomedical applications. As illustrated in Figure 3, these nanofiber scaffolds provide several advantages, including an extensive interface for cell adhesion and proliferation due to their significant ratio of surface area to volume, an important feature for applications such as wound healing and tissue engineering.

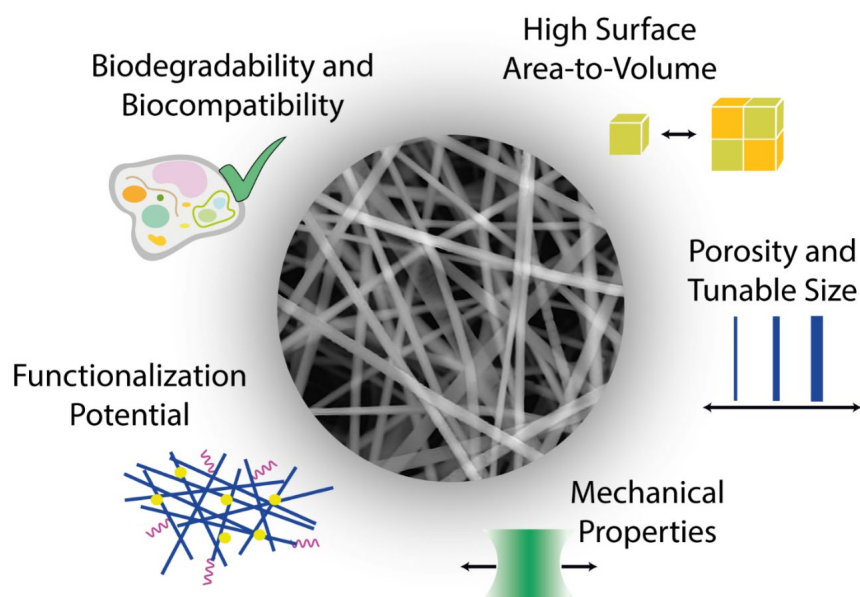


Figure 3: Key Properties of Electrospun Nanofibers. At the center is shown a representative Scanning Electron Microscopy (SEM) image that depicts the fibrous network of electrospun nanofibers. Surrounding the central image are the primary characteristics that define the versatility of electrospun nanofibers. These include a high surface area-to-volume ratio, controllable porosity and fiber dimensions, biodegradability and biocompatibility, tuneable mechanical properties, and a versatile potential for functionalization.

The ability to control dimensions and porosity through the electrospinning process allows for the creation of scaffolds tailored to different needs; for example, larger pore sizes facilitate vascularization in bone tissue engineering, whereas smaller pores are better suited for applications in skin regeneration [79]. Similarly, their mechanical properties are highly tuneable, providing appropriate structural support while maintaining flexibility or rigidity as needed. Furthermore, by integrating a diverse range of bioactive molecules, these scaffolds can be optimized to improve their overall biological functionality. The choice of biodegradable and biocompatible polymers, ensures that these scaffolds integrate seamlessly with host tissues and degrade naturally over time, negating the need for surgical removal. Another critical advantage is their biomimetic nature, as the nanofibrous architecture closely mimics the extracellular matrix, providing an environment conducive to cell adhesion, proliferation, and differentiation [80].

In this context, electrospun nanofibers exemplify how nanostructured materials can be tailored to address specific biomedical challenges, offering a platform that integrates functional versatility, biomimicry, and sustainability. Nevertheless, addressing the complex challenges posed by antibiotic-resistant infections requires a broader, multidisciplinary approach.

1.5 Hypothesis and Aims

This thesis aims to address critical challenges in the field of antibacterial treatments through the investigation of three innovative systems. This research is driven by the growing concern over the increasing resistance of bacteria to commonly available antibacterial strategies, highlighting the urgent need to identify and develop alternative therapeutic approaches capable of ensuring effective treatments in the future.

The central hypotheses supporting this research are based on the premise that the integration of new materials resulting from novel fabrication techniques can significantly enhance antibacterial efficacy, promote sustainability, and enable controlled drug delivery mechanisms. Tuneable properties and different application can be obtained controlling their peculiar properties at the nano and microscale. It is thus hypothesized that the synergistic combination of nitrogen-doped titanium dioxide nanostructured materials and gold nanorods can substantially improve the generation of reactive oxygen species when activated by visible light, in the context of photodynamic therapy. This enhanced generation is expected to increase antibacterial activity through the effective photodegradation of various molecular antibacterial targets, such as proteins, lipids, and DNA, thereby offering a promising strategy for photodynamic therapy and alternative approaches to traditional antibiotic treatments.

Additionally, it is here proposed that lignin-based nanofibers may constitute new sustainable biomaterials. The focus is on optimising water-based process which allow for the control morphological and physicochemical properties. The presented approach aims at exploiting the large number of lignin advantages in biomedical applications.

Furthermore, the potential of polymeric microparticles produced via electrospraying is explored to achieve sustained release of ciprofloxacin, a broad-spectrum antibiotic. The hypothesis suggests that the electrospraying technique can create uniform microparticles with precise control over drug release kinetics, thereby maintaining effective therapeutic levels over extended periods. This approach aims to address the persistent challenge of chronic bacterial infections and reduce the emergence of antibiotic resistance.

Building on these hypotheses, the objective of this thesis is to develop and characterize different antibacterial systems with a focus on the analysis of their morphological and structural characteristics. The aim is to understand how aspects such as shape, size, and surface-related features influence antibacterial mechanisms. This understanding will help clarify the relationship between material properties and their biological performance, providing insights into how these characteristics contribute to antibacterial activity. The ultimate goal is to generate knowledge that can guide the design of biomaterials optimized for multitarget antibacterial applications.

These objectives will be pursued through key strategies, including photodynamic therapy, the use of micro- and nanoparticles or composite nanostructured systems, and the application of biomaterials as three-dimensional matrices. Through these investigations, the thesis aspires to contribute to the advancement of multifunctional antibacterial strategies, offering innovative therapeutic alternatives to traditional antibiotics and addressing the pressing issue of antimicrobial resistance in biomedical treatments.

Chapter 2

Experimental Approaches

In this chapter, the key experimental methods employed in this research are summarized, along with explanations of the basic principles behind each technique. Fundamental details regarding materials production and characterization are provided, which will be further elaborated upon in the results sections.

2.1 Synthesis and Production of Nanostructured Materials

2.1.1 Synthesis of Nitrogen-Doped Titanium Dioxide (N-TiO₂)

The synthesis of TiO₂ can be achieved through various methods, each offering distinct advantages in controlling the properties of the material, including the size of the particles, surface area, crystallinity, and morphology [81]. Common synthesis methods include sol-gel, hydrothermal, and chemical vapor deposition, among others [69,82]. The sol-gel method is particularly popular due to its ability to produce highly pure and homogeneous TiO₂ nanoparticles with controllable size and morphology [81,83]. This process involves the hydrolysis of titanium alkoxides or other titanium precursors in a solvent, followed by their condensation, which ultimately leads to the development of a network, similar to a gel, that can be dried and calcined to yield crystalline TiO₂ [84]. The conditions of the sol-gel process, including pH levels and temperature, but also the concentration of the precursor, can be finely tuned to manipulate the final properties of the TiO₂ [85]. An additional crucial advantage of this synthetic approach is its straightforward methodology, which allows the avoidance of hazardous and toxic reagents, thus contributing to the sustainability of the process by offering a greener alternative.

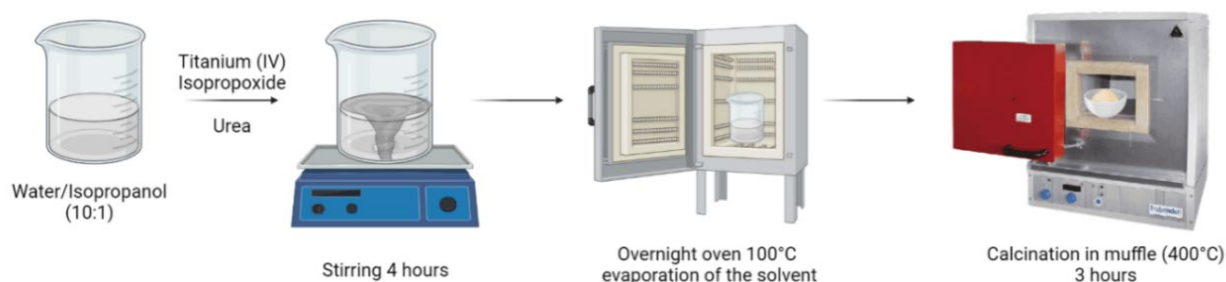


Figure 4: **Schematic representation of the synthesis protocol for N-doped TiO_2** , based on the experimental procedure reported in [83]. A water/isopropanol mixture (10:1) is used as the solvent, with titanium(IV) isopropoxide as the titanium precursor and urea as the nitrogen dopant. The solution is stirred at room temperature for 4 hours, then the solvent is allowed to evaporate overnight at 100°C. The dried material is then calcined at 400°C for 3 hours in a muffle furnace. Created with Biorender.com.

Nitrogen-doped titanium dioxide (N- TiO_2) was synthesized following a sol-gel approach (Figure 4), which is well-known for producing uniform and highly crystalline metal oxide nanoparticles, following the procedure reported in [83]. The process began with the hydrolysis of titanium (IV) isopropoxide (TTIP) in a solution of water and isopropyl alcohol at a 10:1 ratio. Isopropyl alcohol served as the solvent to ensure the even dispersion of TTIP, while water acted as the hydrolyzing agent to fully convert TTIP into a titanium hydroxide network, a precursor to TiO_2 . To introduce nitrogen into the TiO_2 structure, urea was added to the solution, with a controlled titanium-to-nitrogen molar ratio of 1:6. Urea was selected for this purpose as it is a non-toxic compound and can serve as an effective source of nitrogen. During the subsequent calcination process, urea undergoes thermal decomposition, releasing ammonia in situ [86], which provides the nitrogen ions, which can be more readily incorporated into the TiO_2 lattice [81,87]. The resulting mixture underwent a vigorous stirring process to ensure a homogeneous distribution of the precursors, allowing the formation of a sol-gel phase through hydrolysis and polycondensation reactions. This step was followed by drying the slurry in an oven at 100 °C overnight to evaporate the solvent. Subsequently, the material was calcined at 400 °C for 3 hours, a critical step that not only facilitates crystallization but also promotes the incorporation of nitrogen into the lattice, resulting in an efficient doping process. The synthesized nanoparticles were then purified by sonication and washing in a sequence of solvents with decreasing polarity: water, ethanol, and acetone. This purification process helped to remove residual reactants and by-products, ensuring high-purity N- TiO_2 nanoparticles. For comparison, undoped TiO_2 was prepared following the same procedure except for the addition of urea, allowing to evaluate the effects of doping.

2.1.2 Synthesis of Gold Nanorods (AuNRs)

Gold nanoparticles (AuNPs) are nanoscale materials that have garnered great interest in various fields because of their remarkable features and versatility. These particles, typically in the range of 1-100 nm in diameter, exhibit distinctive optical, electronic, and catalytic characteristics that make

them invaluable in applications such as drug delivery, imaging, and photothermal therapy [88,89]. AuNPs can be synthesized through a variety of chemical methods, each tailored to produce nanoparticles of specific shapes, sizes, and functionalities. The chemical reactions, typically involve the reduction of gold salts, such as gold chloride (HAuCl_4), with the use of stabilizing agents that prevent agglomeration and control particle morphology. Moreover, AuNPs can be synthesized in different forms, including spheres, rods, cubes, and more complex structures, depending on the experimental conditions such as the choice of reducing agent, stabilizer, temperature, and reaction time [90]. Among these, AuNRs attract significant attention because of their distinctive shape and optical characteristics. AuNRs exhibit strong surface plasmon resonance (SPR) characteristics, allowing them to absorb and scatter light at specific wavelengths. This tuneable optical response is primarily due to their elongated structure, enabling the manipulation of the plasmonic resonance frequency by adjusting their aspect ratio [91,92]. The synthesis of AuNRs is a critical process that determines their structural and functional properties, and several methods have been developed to achieve the desired shape and size.

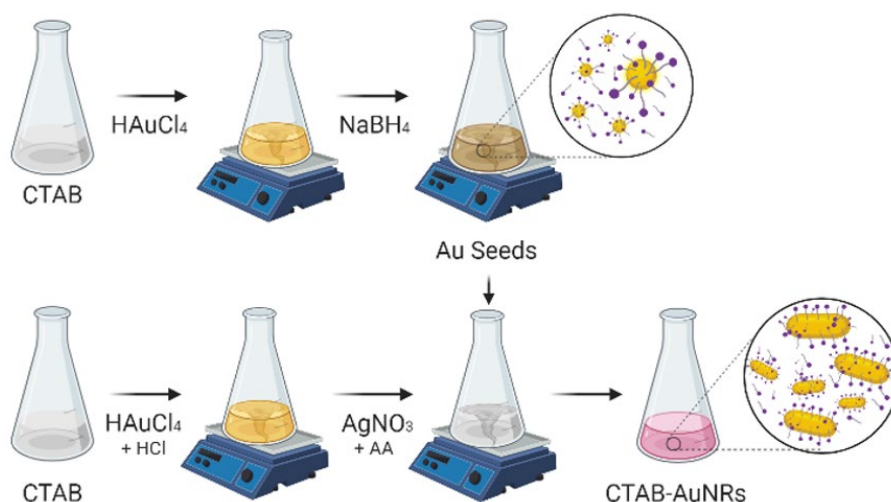


Figure 5: Schematic representation of the synthesis of CTAB-stabilized gold nanorods (CTAB-AuNRs), based on the experimental procedure reported in [93]. Gold seeds are synthesized by reducing HAuCl_4 in a CTAB solution with NaBH_4 . For nanorod growth, a CTAB and HAuCl_4 solution is treated with HCl , followed by the sequential addition of silver nitrate (AgNO_3), ascorbic acid (AA), and gold seeds as a nucleation template. The formation of CTAB-AuNRs is confirmed by the characteristic violet coloration of the solution. Created with Biorender.com.

Among the various methods, the "seed-mediated" growth technique is particularly notable as it allows for precise control over the aspect ratio of the nanorods. This method consists of two primary phases: the preparation of small gold seed particles and their subsequent introduction into a growth solution where they serve as nucleation sites for the anisotropic elongation of nanorods [90]. Specifically, in our experimental setup shown in Figure 5, AuNRs were synthesized following a modified seed-mediated protocol as previously described in the literature [93,94]. The process began with the creation of gold seeds by stirring a solution of 0.25 mM HAuCl_4 in 0.1 M cetyltrimethylammonium bromide

(CTAB) which is a cationic surfactant that stabilizes the particles and controls their morphology during the growth. To this mixture, 300 μL of 0.01 M sodium borohydride (NaBH_4) in ultrapure water were added. NaBH_4 acts as a strong reducing agent, facilitating the rapid reduction of gold ions to form seed particles. This reaction is crucial, as it generates the initial nanoparticles that will later grow into AuNRs. Subsequently, once the seeds were prepared, the growth solution was prepared by mixing 0.025 mmol of HAuCl_4 with 950 μL of 1 M hydrochloric acid (HCl) in 50 mL of 0.1 M CTAB [94]. HCl facilitates the ion exchange process, where bromide ions (Br^-) from CTAB are exchanged with chloride ions (Cl^-) from HCl . This ionic exchange is significant because chloride ions have a lower affinity for binding to gold surfaces compared to bromide ions, which allows for better control over the growth process and morphology of the nanorods. Then, to this solution, 600 μL of 0.01 M silver nitrate (AgNO_3), 400 μL of 0.1 M ascorbic acid, and 120 μL of the freshly prepared gold seeds were added [95]. Finally, the mixture was then incubated at 28°C for 18 hours, during which the gold nanorods formed, stabilized, and were shaped by the CTAB surfactant.

2.2 Production of Nanofibers and Microparticles

A wide variety of techniques have emerged to produce biomaterials, each with its advantages and limitations. Among these, electrospinning has become particularly notable for producing nanofibrous structures that mimic the intricate architecture of natural tissues, but also other materials, such as microparticles, while maintaining favourable properties such as simplicity, cost-effectiveness, and scalability [96,97].

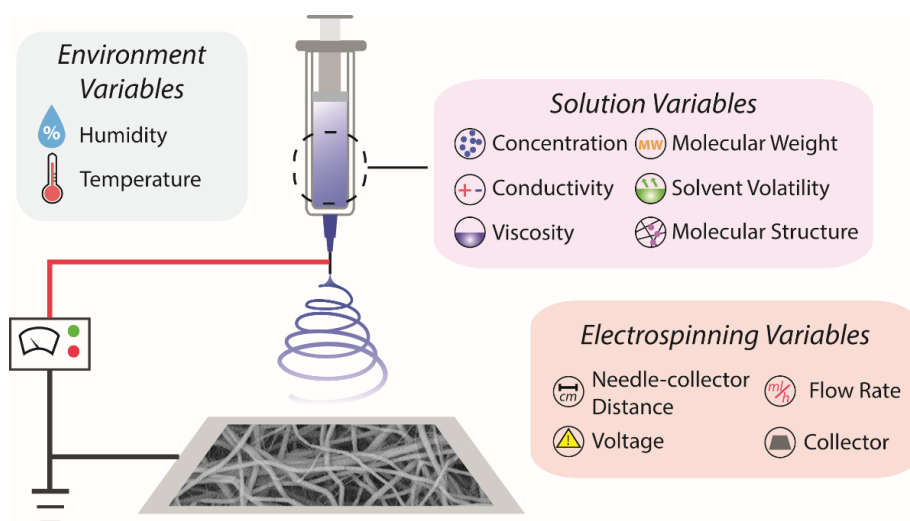


Figure 6: Schematic representation of the electrospinning process, highlighting the key variables influencing it. Solution variables include concentration, molecular weight, conductivity, viscosity, solvent volatility, and molecular structure, which affect jet stability and fiber morphology. Electrospinning variables include needle-to-collector distance, voltage, flow rate, and collector type, which control jet trajectory and fiber deposition. Environmental variables, such as temperature and humidity, influence solvent evaporation and fiber solidification. The diagram highlights the interplay of these factors in producing nanofibers through electrospinning. Illustration recreated based on [98], with adjustments.

The typical electrospinning setup is shown in Figure 6. A syringe is filled with a polymer solution, and its needle is attached to a high-voltage power source. The syringe is mounted on a pump that allows precise control of the solution flow rate. When a high voltage is applied to the needle, the polymer solution becomes charged due to the generated electric field. As the applied voltage rises, the resulting electrostatic forces surpass the surface tension, leading to the elongation of the solution and the formation of what is known as *Taylor cone*. This step is crucial and is significantly affected by the solution's viscosity and environmental factors, including temperature and humidity. Once these forces reach a magnitude that exceeds the solution's surface tension, a thin polymer jet is expelled and undergoes elongation while moving toward the grounded collector [99,100]. During this process, solvent evaporation occurs, leading to the solidification of the solution into continuous nanofibers. The interplay between liquid surface tension, electrostatic repulsion, and the viscosity of the polymer solution determines the diameter and morphology of the resulting fibers. The versatility of this technique enables researchers to precisely modify essential factors such as polymer concentration, solution viscosity, applied voltage, and the distance between the nozzle and the collector, enabling control over fiber morphology, diameter, and orientation [101–103].

As previously stated, the electrospinning process critically depends on solvent evaporation, as this phenomenon directly influences the solidification of the polymer jet upon its ejection from the spinneret. Rapid solvent evaporation facilitates fiber formation and the development of a porous network, which are essential for defining the scaffold's key properties, such as porosity, mechanical strength, and surface morphology [101,104]. Volatile solvents are often used in this process because they evaporate quickly, promoting the rapid solidification of the fibers and ensuring a stable scaffold structure.

Among the various techniques derived from traditional electrospinning, two methods of particular relevance to this research are waterborne electrospinning and electrospraying. Both approaches leverage the fundamental principles of electrospinning while introducing distinct characteristics that make them suitable for specific applications.

2.2.1 Waterborne Electrospinning

Given the significant toxicity of the most commonly used organic solvents for electrospinning, such as 1,1,1,3,3,3-hexafluoroisopropanol (HFIP), dimethylformamide (DMF), and dichloromethane (DCM), finding greener and more sustainable alternatives is of great importance today. Not only are these solvents often expensive and pose serious environmental risks, but they can also leave harmful residual impurities in the final biomaterial, underscoring the need for safer and more cost-effective solutions in the field [105,106]. Waterborne electrospinning is based on the use of water as the sole

solvent, providing notable advantages in terms of biocompatibility and environmental sustainability. However, the lower viscosity and higher evaporation rate associated with aqueous solutions require careful optimization of processing parameters [107]. For instance, the polymer solubilization phase is crucial, as the ability of the polymer to dissolve effectively in water influences the electrospinning process. Additionally, controlling environmental parameters such as humidity and temperature is essential to facilitate the efficient evaporation of water [104,105,108,109]. High humidity may hinder the evaporation process of the polymer jet, potentially resulting in poor fiber development. To ensure the jet is sufficiently dry upon reaching the collector, it is crucial to optimize the needle-to-collector distance, thereby promoting the formation of uniform, continuous nanofibers [100]. Within this thesis, lignin blended with PEO were crafted into nanofibers using a water-based electrospinning technique, marking a significant step toward sustainable materials science. This process was optimized to completely avoid the use of organic solvents, showcasing an innovative approach to harnessing lignin's potential.

2.2.2 Microparticles Production by Electrospraying

Electrospraying is a technique similar to electrospinning, with the main difference being that it produces fine droplets or polymeric microparticles instead of continuous fibers [110,111]. Altering the solvent or adjusting specific preparation parameters, including solution viscosity and flow rate, can influence the electrospinning process to control particle size and morphology effectively. The equipment utilized for electrospraying closely resembles that of conventional electrospinning, consisting of a syringe containing the polymer solution, which is linked to a high-voltage power source to generate an electric field at the tip of the nozzle. As the solution exits, the electric forces act on the liquid, breaking it into charged droplets that are propelled toward a grounded collector. One of the key advantages of electrospraying is its ability to create uniform particles with a controlled size, making it particularly useful for applications in drug delivery, where precise dosing and controlled release are critical. Additionally, the technique allows for the encapsulation of active substances, such as drugs or bioactive compounds, directly within the particles, enhancing their effectiveness in biomedical applications [112–114]. The characteristics of the generated particles, including their size and morphology, are strongly affected by process variables like the applied voltage, flow rate, and the distance between the nozzle and the collector. Modifying these parameters enables researchers to fine-tune the structural properties to suit particular application requirements [98]. Moreover, electrospraying allows for the incorporation of active substances, such as drugs or bioactive compounds, directly into the particles, enhancing their functionality for biomedical applications.

2.4 Spectroscopic Techniques

2.4.1 UV-Vis Absorption Spectroscopy

Ultraviolet-visible (UV-Vis) absorption spectroscopy is an essential analytical method used to explore the electronic transitions of molecules by measuring how much ultraviolet (UV) and visible (Vis) light a sample absorbs. The core principle of this technique is described by the *Lambert-Beer law*, which establishes a relationship between the absorbance (A) of a substance and key parameters such as its concentration (c), the optical path length (l), and the molar absorptivity coefficient (ϵ):

$$A = \epsilon \cdot c \cdot l$$

This correlation allows for the quantification of the concentration of substances and gain insights into their electronic structure based on their light absorption characteristics at specific wavelengths. In this thesis work, UV-Vis absorption spectroscopy has been extensively used to monitor and characterize the behaviour of molecular agents in solution [115].

Additionally, reflectance measurements using an integrating sphere have been employed to characterize solid materials, particularly those that are insoluble or have limited solubility in water. An integrating sphere is a spherical device lined with highly reflective material, designed to capture diffuse reflectance from a sample. This configuration allows for effective analysis of powders and other solid materials, providing a comprehensive understanding of their optical properties.

2.4.2 Dynamic Light Scattering and Zeta-Potential

Dynamic light scattering (DLS) is a powerful technique widely used for analyzing size and distribution of particles in colloidal suspensions [116]. The method measures the Brownian motion of particles in a liquid and, from the fluctuations in scattered light intensity, calculates their hydrodynamic diameter. [117] In this context, Z-average is the intensity-weighted average of the particle sizes, providing a detailed evaluation of the particle size distribution in the sample. In addition to particle size analysis, the zeta potential, which indicates the surface charge of particles in suspension, was also measured. Zeta potential is determined by analyzing the electrophoretic mobility of particles in an applied electric field, providing valuable information on colloidal stability [118]. Typically, higher absolute values of zeta potential indicate stronger electrostatic repulsion between particles, reducing aggregation and enhancing stability. In this thesis, DLS was employed to evaluate the size and polydispersity index (PDI) of gold nanorods (AuNRs) and small unilamellar vesicles (SUVs) dispersed in aqueous solutions.

2.4.3 Infrared Spectroscopy

Fourier-transform infrared (FTIR) spectroscopy was utilized in this thesis, employing both transmission and attenuated total reflectance (ATR) modes. The primary difference between these two methods lies in their interaction with the sample. Transmission FTIR requires the sample to be sufficiently transparent to allow the infrared radiation to traverse through it, enabling the analysis of the absorbed frequencies [119]. In contrast, ATR-FTIR relies on measuring the infrared light reflected at the interface between an Internal Reflection Element (IRE) and the sample, which makes it particularly suitable for the analysis of opaque or thick samples. The ATR technique leverages the phenomenon of the evanescent wave, a non-propagating electromagnetic wave generated at the interface where total internal reflection occurs within the IRE. This evanescent wave penetrates a few microns into the sample, interacting with its molecular components and enabling surface-sensitive measurements [119].

FTIR detects molecular vibrations, including stretching and bending modes of various chemical bonds, providing a robust tool for the identification and characterization of the chemical composition of materials. It allows both qualitative recognition and quantitative analysis of characteristic vibrational peaks corresponding to specific functional groups [120]. When analyzing liquid samples, solvent selection is critical to minimize spectral interference. Deuterated solvents, such as deuterium oxide (D_2O), are often utilized to suppress solvent-related absorption bands. Furthermore, calcium fluoride (CaF_2) windows are employed in such cases thanks to their high transparency within the mid-infrared region, ensuring optimal performance in transmission mode [119,120].

2.4.4 Fluorescence Spectroscopy

Fluorescence spectroscopy is an analytical technique based on the light emitted by a material following the absorption of photons. The fundamental principle behind this technique involves exciting electrons in a fluorophore, a molecule that fluoresces, using a light source at a specific wavelength (excitation wavelength). These excited electrons then relax to a lower energy state, emitting light at a longer wavelength (emission wavelength) in the process [121]. The detected light, emitted by the fluorophore, undergoes analysis to reveal details about its molecular surroundings and interactions. The theory of fluorescence spectroscopy rests on the concepts of electronic transitions and photophysical processes [114].

2.4.5 Two-Photon Excitation Fluorescence Microscopy

Two-photon excitation fluorescence microscopy is a powerful imaging technique that significantly enhances the ability to visualize fluorescent samples at high spatial resolution, which may be

intrinsically fluorescent or labeled with fluorophores. For the study of three-dimensional samples, two-photon excitation microscopy represents a unique tool, enabling optical sectioning with minimal invasiveness and deeper sample penetration [122].

In this thesis, two-photon excitation microscopy is employed to visualize lignin nanofibers, leveraging their intrinsic autofluorescence properties to provide detailed insights into their structural organization and potential applications in materials science. This imaging technique enhances fluorescence imaging through the simultaneous absorption of two photons to excite fluorophores. Utilizing near-infrared light and femtosecond pulsed lasers, this method achieves the high peak power necessary for efficient two-photon absorption, a concept first proposed by Maria Göppert Mayer in her mid-20th century research [123,124]. The technique relies on nonlinear optical effects, concentrating excitation at the laser focal point and minimizing out-of-focus excitation. This results in high-resolution images suited for examining fine details within 3D materials while reducing scattering contributions. Furthermore, two-photon excitation allows for the activation of molecules that absorb in the ultraviolet range, making it particularly advantageous for observing the autofluorescence of many natural systems that often absorb light in this spectrum [125].

2.4.7 Fluorescence Lifetime Imaging Microscopy

FLIM was utilized as a primary analytical method to investigate the distinctive autofluorescence characteristics of lignin-derived nanofibers and to analyze their structural interactions with the surrounding matrix. By utilizing the phasor approach, introduced by Enrico Gratton and Michelle Digman [126], it became possible to measure and analyze the fluorescence lifetime of these nanofibers without the need for external staining, providing a detailed understanding of their photophysical behaviour and molecular organization. This foundational context highlights the relevance of FLIM and the phasor plot approach in the study of complex, heterogeneous systems like lignin-based nanomaterials.

Unlike intensity-based fluorescence imaging, which is influenced by fluorophore concentration and photobleaching, FLIM measures the temporal properties of emitted light, which are independent of fluorophore concentration and photobleaching, making it particularly suited for studying heterogeneous systems [127].

The phasor approach represents a powerful method for analyzing fluorescence lifetime data acquired through FLIM. This technique transforms the decay data from each pixel of the fluorescence image into a graphical representation known as the phasor plot. The mathematical foundation for the phasor transformation was originally introduced by Gregorio Weber [128] to analyze heterogeneous systems and later adapted into its graphical form by Jameson and collaborators [129]. This technique's

flexibility stems from its capability to handle data acquired in both the time and frequency domains. In the context of time-correlated single-photon counting (TCSPC), photon arrival time histograms for each pixel are transformed into the frequency domain through the application of a Fourier transform [130]. Subsequently, the phasor coordinates, denoted as G and S , are determined as follows:

$$G = \int_0^{\infty} I(t) \cos(\omega t) dt; \quad S = \int_0^{\infty} I(t) \sin(\omega t) dt$$

where $I(t)$ is the fluorescence decay at time t , and ω is the angular frequency. These coordinates are plotted on a polar diagram, referred to as the phasor plot, which provides an intuitive representation of the fluorescence lifetime properties. The phasor plot has several key advantages. For single-exponential decays, the coordinates fall on a semicircular arc called the "*universal circle*" [131]. The position along this arc corresponds to the fluorescence lifetime, with shorter lifetimes appearing near $(G,S) = (1,0)$ and longer lifetimes near $(G,S) = (0,1)$. The workflow of FLIM and its integration with the phasor plot approach is illustrated in Figure 7, with a step-by-step explanation of how fluorescence lifetime data is processed and visualized.

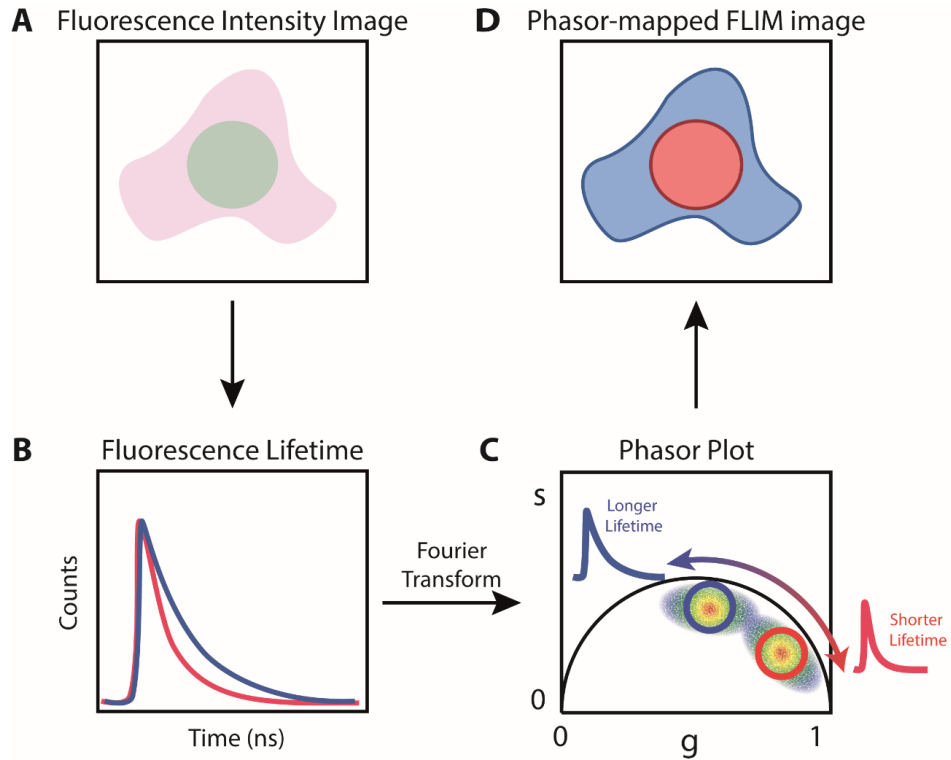


Figure 7: Illustration of the process for generating phasor-mapped FLIM images. (A) Fluorescence intensity image is acquired, representing the spatial distribution of fluorescence. (B) Profile of fluorescence decay are extracted for individual pixel, with the graph showing the intensity signal versus time. (C) These temporal decay profiles are transformed into the frequency domain using a Fourier transform, resulting in a phasor plot, in which the position of each point corresponds to the fluorescence lifetime of a given pixel. The position on the phasor plot correlates with fluorescence lifetimes: longer lifetimes are mapped closer to the origin (0,0), while shorter lifetimes are located towards (1,0) [132]. (D) The phasor information is mapped back onto the spatial image to create the final FLIM image, where lifetime variations are color-coded. Illustration inspired by and redrawn from [130,133].

In Figure 7A, a fluorescence intensity image is shown, representing the spatial distribution of fluorescence signal intensity within the sample. In Figure 7B depicts fluorescence lifetime decay curves, which describe the intensity of emitted light as a function of time following excitation. The decay behaviour varies depending on the sample, exhibiting either a single-exponential or a multi-exponential profile [134]. In either case, the data undergoes a Fourier transform to convert it into the frequency domain, as demonstrated in Figure 7C, generating the phasor plot. In this diagram, single-exponential decays are positioned along the universal circle, with shorter lifetimes located on the right and longer lifetimes on the left [135]. Multi-exponential decays, which occur in heterogeneous systems or due to overlapping lifetimes, are represented as points within the semicircle. The phasor plot provides a model-free approach for analyzing fluorescence lifetime distributions, avoiding the need for pre-assumed fitting models and allowing direct visualization of lifetime heterogeneity across the sample [130,136] Finally, Figure 7D integrates this information into a spatially resolved FLIM map, where each pixel is color-coded according to its lifetime properties. This enables the visualization of molecular interactions and environmental variations directly within the sample.

2.5 Fluorescent Probes

Fluorescent probes are molecules that emit fluorescence upon excitation and are widely used to investigate the properties of the samples under study [121]. These molecules act as sensitive reporters, providing detailed information on the structural, dynamic, and physicochemical characteristics of the environment they interact with. By binding to or partitioning within specific regions of the sample, fluorescent probes enable the detection and quantification of changes in properties such as polarity, phase behaviour, and molecular organization [137].

In this thesis, two fluorescent probes have been employed to stain lipid-based samples, to explore lipid organization and dynamics, with a focus on elucidating the effects of photooxidation damage, as detailed in the following sections.

2.5.1 C-11 BODIPY^{581/591} Dye

C-11 BODIPY^{581/591}, a specific fluorescent probe from the BODIPY (Boron-DiPyrromethene) family, plays a crucial role in fluorescence applications due to its exceptional photophysical properties. BODIPY dyes are recognized for their high quantum yield, excellent photostability, and narrow emission bands, which make them particularly suitable for fluorescence microscopy and spectroscopy. These dyes have been introduced as alternatives to commonly used probes such as fluorescein and rhodamine. Typically, BODIPY dyes emit light in the green region (450-500 nm), and structural modifications, such as elongation of the main core (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene), result

in a red shift of their emission spectra [138,139]. C-11 BODIPY^{581/591} is a fluorescent probe widely used as sensor for detecting lipid peroxidation and oxidative processes within lipidic systems. It is capable of integrating into lipid bilayers, making it an excellent tool for investigating oxidative events in lipid environments. This is possible thanks to its molecular structure, which includes a typical core derived from the BODIPY (BDP) dye family. This core is linked to a phenyl moiety via a diene bond that serves as a resonance bridge within a conjugated system, as illustrated in Figure 8A. Additionally, the BDP core is attached to a fatty acid tail (highlighted in orange) which imparts its lipid-mimetic properties [140].

Upon interacting with free radicals, the fluorescence undergoes a pronounced shift from red to green emission, driven by the loss of the phenyl group during diene linker oxidation (Figure 8B). This results in strong green fluorescence at 520 nm, while the non-oxidized form emits at 595 nm. Importantly, these interactions occur with minimal impact from environmental changes, ensuring reliable results in lipid peroxidation studies [141].

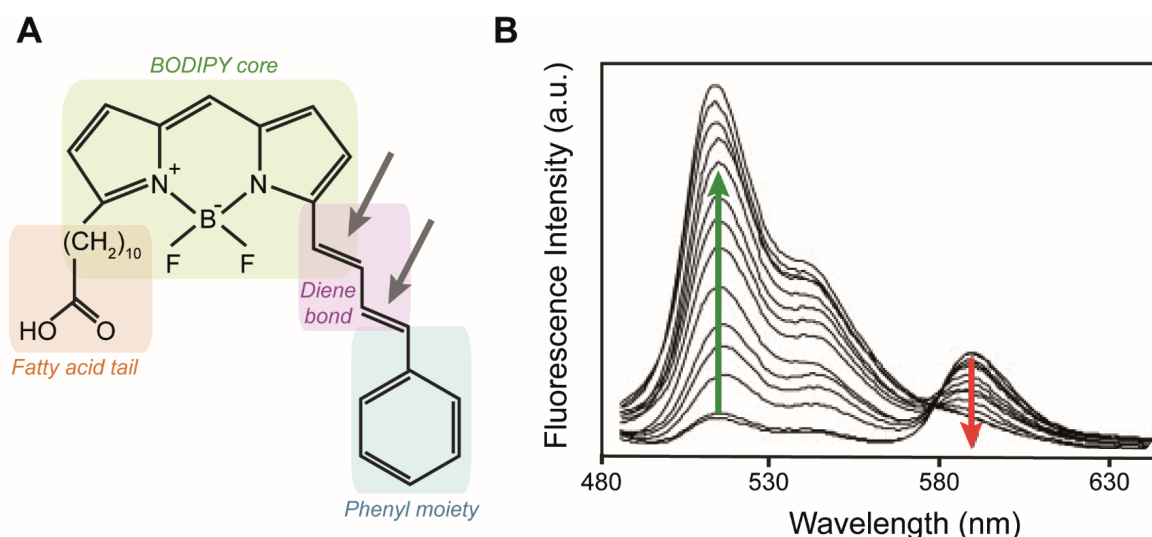


Figure 8: Chemical structure and fluorescence emission spectra of the C11-BODIPY^{581/591} molecule. (A) The chemical structure highlights key features, including the fatty acid tail, the BODIPY core, and the phenyl moiety. Arrows indicate the diene bond, which is susceptible to oxidative modification. (B) Fluorescence emission spectra under oxidative conditions illustrate a shift characterized by a decrease in fluorescence intensity in the red region (~590 nm, red arrow) and a corresponding increase in the green region (~520 nm, green arrow), reflecting oxidation-induced spectral changes. Illustration adapted and redrawn from [142] with modifications.

The diene interconnection is the most likely target of ROS, and its rupture disrupts the conjugated system between the BODIPY core and the phenyl group. Consequently, once oxidized, C-11 BODIPY^{581/591} returns to the spectral properties characteristic of the original BODIPY core, which exhibits pronounced fluorescence in the green region [142,143]. According to Stockert et al., although the green fluorescence can be affected by various oxidative processes that produce non-fluorescent compounds or result in additional photobleaching reactions, the decline in red fluorescence is regarded

as the most dependable marker for lipid peroxidation [144]. In its reduced, unoxidized form, C-11 BODIPY^{581/591} exhibits a fluorescence emission peak at 594 nm. When subjected to oxidative stress, particularly through the disruption of its diene bond, the emission spectrum undergoes a notable shift toward greener wavelengths, peaking around 520 nm. This spectral shift reflects alterations in the molecule's electronic structure caused by the breakdown of its conjugated system, leading to enhanced green fluorescence [140,145]. The diene group is especially prone to oxidation by free radicals, making C-11 BODIPY^{581/591} a highly effective probe for detecting oxidative stress in lipid bilayers. Additionally, the behaviour of C-11 BODIPY in the lipid bilayer is further influenced by its propensity to form excimers, dimeric complexes that can significantly modify the probe's fluorescence properties. Excimer formation occurs when C-11 BODIPY molecules are in close spatial proximity, often at high local concentrations, resulting in changes to their characteristic emission profiles [146,147].

2.5.2 Laurdan Dye

Laurdan, (6-Lauroyl-2-dimethylamino-naphthalene), is a fluorescent dye belonging to the Dansyl dye family, first synthesized by Weber. It is renowned for its distinct spectral characteristics and its widespread application in membrane studies [148,149]. Figure 9A illustrates the chemical structure of Laurdan, highlighting the presence of a naphthalene moiety. Laurdan's unique photophysical properties are closely linked to its dipole moment, which plays a crucial role in its sensitivity to environmental changes. This dipole moment arises from the charge separation between the 6-carbonyl and 2-dimethylamino groups [150]. Such a structural feature enables Laurdan to effectively respond to variations in the polarity of its surroundings. Upon excitation, changes in its dipole moment induce solvent dipole reorientation, such as that of water molecules. This process, known as dipolar relaxation, leads to a decrease in the probe's excited-state energy, manifesting as a red shift in its emission spectrum. In lipid systems, the dynamics of water molecules is influenced by the lipid phase state [151]. In the liquid-crystalline phase, characterized by its dynamic molecular organization, the orientation of water molecules tends to align with Laurdan's dipole, highlighting their interaction with the surrounding microenvironment [152].

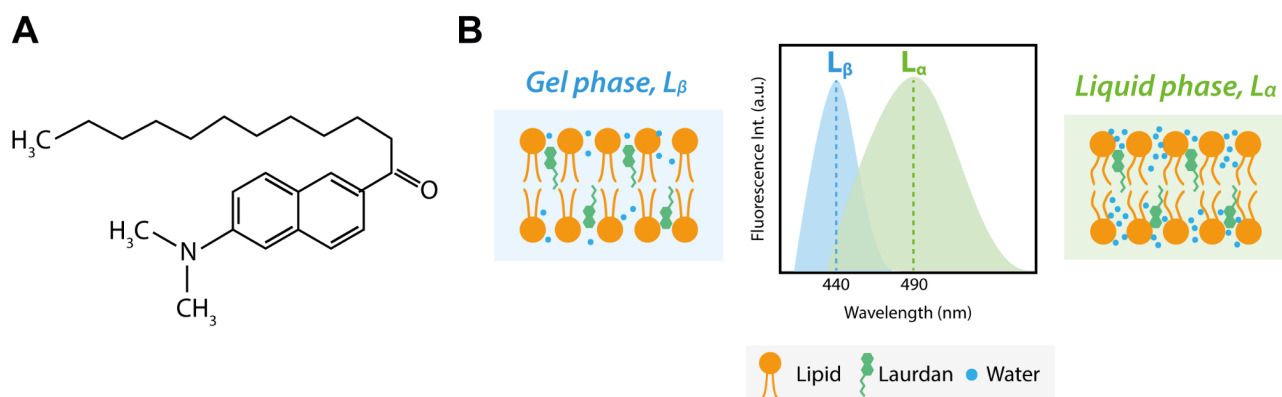


Figure 9: Laurdan molecular structure and fluorescence properties in lipid bilayers. (A) The molecular structure of Laurdan, composed of a naphthalene core and a hydrophobic tail that facilitates its incorporation into lipid bilayers. (A) Laurdan fluorescence emission spectra correlate with lipid packing, order, and hydration: in the gel phase (L_{β}), characterized by tightly packed and highly ordered lipids with low hydration, Laurdan exhibits a blue-shifted emission (~ 440 nm) due to minimal dipolar relaxation. In contrast, because of the increased fluidity in the liquid-crystalline phase (L_{α}), which is associated with disordered lipids and greater water penetration, Laurdan exhibits a red-shifted emission (~ 490 nm), reflecting enhanced dipolar relaxation (B). Illustration inspired by and redrawn from [153].

Figure 9B illustrates the two primary components of Laurdan's emission. In more ordered or gel-like membrane phases, the emission shifts towards the blue region of the spectrum, reflecting a restricted environment that limits dipolar relaxation. In contrast, in fluid or disordered membrane phases, the emission shifts towards the green region, indicating enhanced molecular mobility and a less constrained dipolar relaxation process [154].

This shift can be quantified using the Generalized Polarization (GP) method, which is derived from the ratio of the blue to green fluorescence intensities. Laurdan's utility extends to both bulk studies and advanced fluorescence imaging. In bulk cuvette assays, Laurdan helps in assessing the overall membrane fluidity and hydration by analyzing the shifts in emission spectra under varying conditions. This approach provides a macroscopic view of membrane characteristics, including packing density and water accessibility. In fluorescence imaging, Laurdan allows for spatially resolved observations of membrane properties [155,156]. By visualizing Laurdan-stained samples under a fluorescence microscope, researchers can obtain detailed maps of membrane order-disorder across different cellular or tissue regions. This capability is particularly valuable for studying heterogeneous membrane environments and dynamic processes such as phase transitions and interactions with other biomolecules [157,158]. In summary, Laurdan is an essential tool for probing membrane dynamics due to its spectral sensitivity to environmental changes. Its ability to provide insights into membrane order-disorder transitions, packing density, dipolar relaxation, and hydration makes it a versatile and powerful probe in both bulk assays and fluorescence imaging applications [155]. The theoretical principles underlying Laurdan's fluorescence properties enable a comprehensive understanding of membrane biophysics, essential for advancing our knowledge in cellular and material sciences.

2.6 Electron Microscopy Techniques

Electron microscopy includes various techniques that utilize electron beams to generate high-resolution images of materials at the nanoscale, which are essential for characterizing their morphology, structure, and composition. In this thesis, both scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were employed to analyze microparticles, scaffold nanofibers, and nanoparticles, each providing distinct insights into material properties.

2.6.1 Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) provides detailed three-dimensional images of a sample's surface. This technique operates by directing a focused electron beam to scan the specimen's surface of the specimen, leading to the emission of characteristic X-rays, backscattered electrons, and secondary electrons through interactions with the sample. The resulting outputs are collected to create high-resolution images that reveal surface topography and composition at the nanoscale [159,160]. In this thesis, SEM has been utilized extensively to characterize nanoparticles aggregates, microparticles and scaffold nanofibers. A significant advantage of SEM is its ability to investigate samples in their native state, without requiring complex sample preparation procedures. In some cases, a gold layer coating is applied to the samples before imaging, which can improve electron conductivity and enhance image quality by increasing the emission of secondary electrons. Additionally, when coupled with energy-dispersive X-ray spectroscopy (EDX), SEM allows for the characterization of elemental composition and phase information, providing valuable insights into the materials being studied [159]. Moreover, certain SEM setups allow for imaging in transmission mode, commonly referred to as scanning transmission electron microscopy (STEM). In this mode, a focused electron beam traverses the specimen, enabling the acquisition of detailed information of thin samples. STEM typically achieves higher spatial resolution than conventional SEM due to the smaller electron probe size and the increased interaction volume in thin specimens, which reduces signal overlap and enhances the precision of the imaging [161]. For STEM analysis, samples such as nanoparticles are often deposited on specialized grids coated with carbon films and supported by a fine metal mesh. These carbon-coated grids provide mechanical stability to the sample while allowing the electron beam to pass through the deposited material. STEM employs transmitted electrons collected by dedicated detectors, providing contrast mechanisms such as mass-thickness contrast and diffraction contrast, which allow for the visualization of internal structures with remarkable clarity. This makes STEM particularly valuable for characterizing fine structural details and nanoscale features, offering complementary insights to the surface-focused imaging of traditional SEM [160,161].

2.6.2 Transmission Electron Microscopy (TEM)

Transmission electron microscopy (TEM) achieves higher resolution by transmitting electrons through thin samples, allowing visualization of internal structures and providing detailed information on structural characteristics at the nanoscale [162]. In this thesis, TEM is applied to study nanoparticles, providing insights into their size and shape.

The TEM setup consists of several essential elements. An electron gun produces the electron beam, which is then accelerated to high energies, typically in the range of 100–300 keV, and focused onto the sample using electromagnetic lenses. To allow electrons to pass through with minimal scattering, the specimen should be prepared as an ultrathin section, usually less than 100 nm thick [163,164]. As the electrons are transmitted through the sample, different contrast mechanisms such as mass thickness contrast, diffraction contrast, and phase contrast are used to produce high resolution images. Detectors or fluorescent screens capture the transmitted electrons, translating the interaction into an interpretable image. While sample preparation for TEM is more demanding compared to other microscopy techniques, the unparalleled resolution and ability to visualize internal structures make it an indispensable tool for materials characterization at the nanoscale [165].

2.7 Dynamic Mechanical Analysis (DMA)

In this thesis, dynamic mechanical analysis (DMA) is utilized to characterize the mechanical behaviour of electrospun nanofibers, offering critical information about their potential for biomaterial applications. The mechanical characteristics of these biomaterials play a crucial role in determining their performance under physiological conditions and their compatibility with biological tissues. To perform DMA, nanofiber scaffolds are secured to clamps that apply tension at a controlled rate, allowing for precise measurement of the stress-strain response during elongation [166]. The resulting stress-strain curve, illustrated in Figure 10, reveals essential mechanical characteristics.

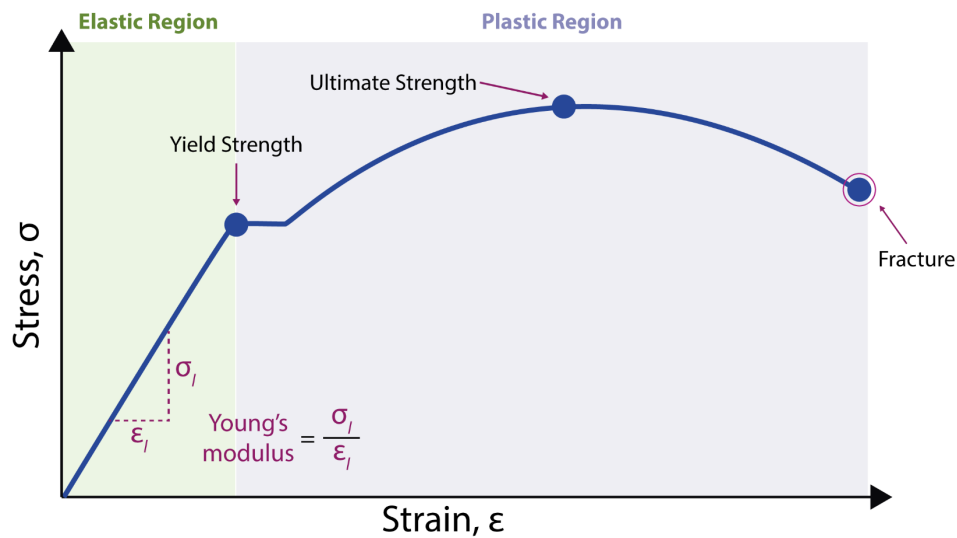


Figure 10: **Stress-strain curve illustrating the mechanical properties of a material under tensile testing.** The graph can be categorized into two main sections: the elastic zone (indicated in green) and the plastic zone (shown in violet). In the elastic region, stress (σ) is linearly proportional to strain (ϵ), defined by Young's modulus, which represents the material's stiffness. The end of the elastic region is marked by the yield strength, indicating the stress at which permanent deformation begins. In the plastic region, the material undergoes irreversible deformation, reaching its ultimate strength, the maximum stress it can withstand. Beyond this point, the material weakens until fracture occurs. Redrawn and adapted from [167,168].

The Young's modulus, also known as the elastic modulus, is a fundamental mechanical property that quantifies the material's stiffness [169]. It is defined as the ratio of stress to strain in the linear elastic region of a stress-strain curve, where the material deforms elastically and returns to its original shape upon the removal of the applied load [170,171]. A higher Young's modulus indicates greater stiffness, a vital property for structural integrity in applications like tissue engineering scaffolds [172]. Additionally, the curve identifies key mechanical thresholds: the yield strength, marking the transition from elastic to plastic deformation; the ultimate strength, representing the maximum stress the material can withstand; and the fracture point, indicating the stress at which the material breaks. These parameters collectively provide a comprehensive understanding of the material's mechanical resilience [173].

Chapter 3

Visible Light Photodegradation of Biomolecules mediated by N-doped TiO₂ and Gold Nanorods

This chapter explores the use of nitrogen-doped titanium dioxide/gold nanorods composites as innovative photosensitisers for photodynamic therapy. Following their synthesis, a detailed spectroscopic and morphological characterization was performed. The study then evaluated their photocatalytic activity under visible light irradiation, focusing on their interaction with key molecular targets such as DNA and lipid membranes.

3.1 Introduction

The use of inorganic photosensitizers, such as TiO₂, holds significant promise in the context of aPDT. As highlighted in Section 1.2, this material shows considerable potential in facilitating the treatment of bacterial infections through the generation of ROS. In this context, it is essential to explore the distinctive electronic and optical properties that characterize it as a semiconductor. Additionally, the optical and electronic properties of AuNRs will be discussed, as they were utilized in this study in combination with N-TiO₂ to enhance photocatalytic efficiency. Lastly, the molecular features of critical antibacterial targets will be explored.

3.1.1 Optical and Electronic Properties of Titanium Dioxide

Semiconductor oxides, such as TiO₂, have emerged as photosensitizers due to their photocatalytic properties. These n-type semiconductors can generate ROS under light exposure, thereby demonstrating significant potential in PDT applications [174]. In this thesis, the focus is directed toward TiO₂, which is widely used in biomedical research for its cost-effectiveness, excellent biocompatibility, and chemical stability [175]. It exists in four different polymorphs, of which anatase and rutile are known to be effective for photocatalytic applications. The photocatalytic activity of semiconductors is primarily governed by their bandgap, which represents the energy gap separating the valence band (VB) from the conduction band (CB). In semiconductors, electrons in the VB are

typically bound to atoms and unable to move freely, whereas electrons in the CB are delocalized and capable of facilitating electrical conductivity (Figure 11).

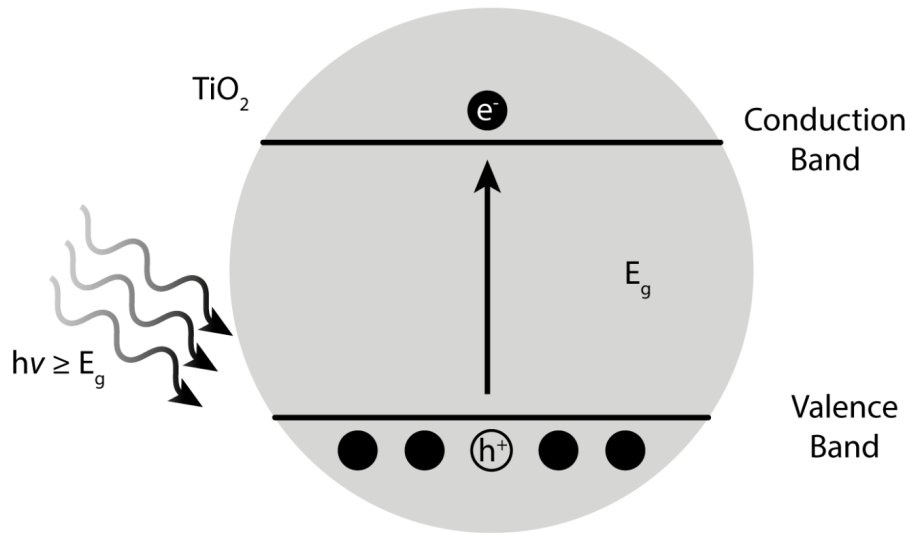


Figure 11: **Schematic representation of charge carrier generation in TiO_2 under photon irradiation.** Excitation occurs when photons possess an energy ($h\nu$) that matches or surpasses the band gap energy (E_g). Due to this energy transfer process an electron (e^-) of the valence band (VB) is able to move towards the conduction band (CB), leaving a corresponding hole (h^+) in the VB. Adapted and redrawn from [176].

When TiO_2 is exposed to light with energy equal to or greater than its bandgap energy, electrons can be excited from the VB to the CB, creating electron-hole pairs (e^-h^+). The initial phase of the photocatalytic process involves the creation of these pairs, which facilitates the production of ROS, such as hydroxyl radicals ($\bullet\text{OH}$) and superoxide anions ($\text{O}_2^{\bullet-}$), which are crucial for their antibacterial activity [82,177]. Despite its advantages, TiO_2 faces challenges due to its activation primarily relying on UV light, which poses limitations in biological systems due to poor tissue penetration and potential phototoxic effects. To overcome these limitations, significant scientific investigations have focused at modifying TiO_2 to extend its light absorption capabilities, particularly into the visible spectrum, thereby enhancing its applicability in aPDT. A wide range of methods has been studied to boost the photocatalytic activity of TiO_2 , including the doping with non-metallic elements, creating heterojunctions with other semiconductors, incorporating noble metal nanoparticles, and implementing surface modifications [178,179].

Doping is a chemical modification which occurs with the introduction of non-metallic elements, such as nitrogen or carbon, into the TiO_2 lattice modifying its electronic structure thereby creating additional energy levels within the bandgap. This allows doped- TiO_2 to absorb longer wavelengths of light, which increases its responsiveness to visible light [180]. In this regard, nitrogen doping can effectively narrow the bandgap from approximately 3.2 eV to around 2.5 eV, enabling visible light absorption. Moreover, constructing heterojunctions with other semiconductor materials can enhance charge separation,

effectively minimizing the recombination of electron-hole pairs, thereby increasing the overall photocatalytic performance [181–183]. For example, the heterojunction of TiO₂ with CdS has shown significant improvement in photocatalytic degradation of organic pollutants, demonstrating the practical benefits of this approach [184]. The integration of noble metals such as gold (Au), platinum (Pt), or palladium (Pd) onto the surface of TiO₂ has been widely researched for its ability to enhance photocatalytic performance [185,186]. These metal nanoparticles act as electron reservoirs that capture excited electrons, thereby preventing their recombination with holes. This process not only increases the lifetime of excited electrons but also enhances the overall charge transfer at the TiO₂ interface. Additionally, the surface plasmon resonance exhibited by these noble metals allows for improved light harvesting in the visible spectrum, further augmenting the production of ROS [187].

3.1.2 Morphological and Plasmonic Properties of Gold Nanoparticles

Gold nanoparticles are a class of nanomaterials that has gained considerable interest owing to their distinct optical, electronic, and catalytic features. These properties are mainly influenced by factors such as particle size, morphology, and an elevated surface-to-volume ratio [188,189]. It is possible to synthesize these nanoparticles in different shapes, including spheres, rods, and cubes [190]. Among these, gold nanorods (AuNRs) exhibit unique optical properties driven by localized surface plasmon resonance (LSPR), which arises from the collective oscillation of conduction electrons in response to incident light [92,191]. Due to their anisotropic, elongated shape, AuNRs display two distinct plasmon modes: a transverse mode along the short axis and a longitudinal mode along the long axis, with the latter extending into the near-infrared (NIR) region. This dual resonance effect allows AuNRs to efficiently absorb and scatter light across a broad spectral range. The tunability of their optical response through aspect ratio modulation enhances their versatility in biomedical applications such as bioimaging, photothermal therapy, and photocatalysis [192,193].

The high surface-to-volume ratio of AuNRs further enhances their reactivity, making them suitable for combining with semiconductor materials like TiO₂ [194–196]. In such composites, AuNRs can act as charge carrier stabilizers, facilitating the separation of photogenerated electron-hole pairs. This is particularly important in photocatalytic processes, in which charge recombination limits efficiency. Moreover, AuNRs can form Schottky heterojunctions at the interface with TiO₂, where the difference in work function between gold and the semiconductor creates a potential barrier. This barrier allows AuNRs to trap excited electrons from TiO₂, preventing recombination with holes and prolonging the lifetime of charge carriers, thereby enhancing the photocatalytic activity [197–199]. Moreover, AuNRs can also participate in plasmonic enhancement mechanisms, where the strong localized electromagnetic fields produced by their plasmon resonance can increase light absorption by the TiO₂

photocatalyst [200]. This, combined with their role in facilitating charge transfer, positions gold nanorods as effective components in advanced nanocomposites designed for photochemical applications [201].

3.1.3 Evaluation of Oxidative Damage in Bacterial Targets

Once the photosensitizing agents have been selected and optimized, it is crucial to evaluate their efficacy by assessing their capacity to oxidize bacterial cells through the generation of ROS. This evaluation is commonly conducted in the literature by studying bacterial cultures in vitro under various conditions.

In this context, Serov et al. have discussed the antimicrobial properties of TiO₂ nanoparticles against various bacteria and fungi, highlighting factors influencing their efficacy, such as particle size, shape, and composition [202]. Another study has reported the effectiveness of TiO₂ nanoparticles prepared via laser ablation and their antibacterial efficacy against various bacterial strains [203]. Additionally, TiO₂-coated surfaces were developed which, under UV irradiation, were shown to effectively inactivate bacterial strains, emphasizing their potential for applications in disinfection and sterilization processes [204]. However, the molecular mechanisms driving conformational changes and structural modifications of target molecules that lead to bacterial damage remain relatively underexplored [205,206].

The present study aims to fill this gap with a molecular biophysical approach by focusing on the study of the structural and conformational changes that oxidative damage generates in key bacterial components, including proteins, DNA and lipids [207]. In Figure 12 our experimental approach is illustrated, emphasizing the three major components of bacterial cells.

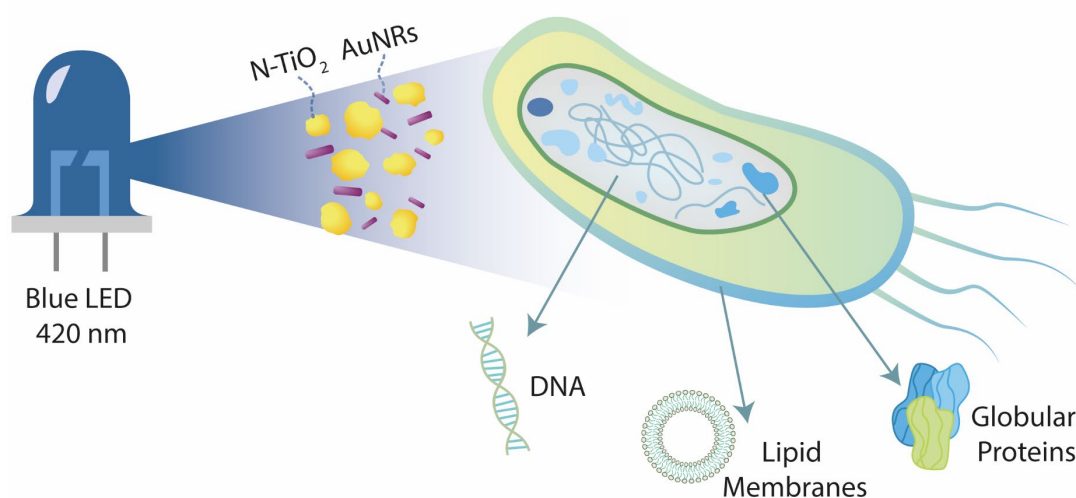


Figure 12: Experimental approach employing photocatalysis against bacterial-like targets. Blue LED light at 420 nm activates N-TiO₂ and AuNRs, resulting in the production of ROS. Model molecules mimicking key bacterial elements including DNA, lipid membranes, and globular proteins are used to investigate the photocatalytic mechanisms involved.

Specifically in this study, Calf-Thymus DNA (CT-DNA) was selected as a model molecule for DNA, because it retains the structural and functional characteristics of native DNA found in living organisms [208,209]. The use of CT-DNA facilitates a focused investigation of the interactions between ROS and the DNA molecule, enabling a detailed observation of various types of oxidative damage. During aPDT, ROS generated by photosensitization are responsible for multiple forms of DNA damage, including oxidative modifications of DNA bases, their excision or release from the double helix, and single- or double-strand breaks [210]. Among these, nucleotides undergo chemical alterations and fragmentation, severely compromising their structural integrity and proper pairing during replication. Notably, guanine is the most susceptible to oxidation due to its low redox potential, rendering it particularly prone to damage. This vulnerability stems from the electronic structure of its purine ring, characterized by a higher electron density. In this context, 8-hydroxy-2'-deoxyguanosine (8-oxo-G) has emerged as a recognized biomarker of DNA oxidation [211]. It is one of the most stable and mutagenic lesions caused by ROS, with the potential to induce erroneous base pairing with adenine during DNA replication [212,213]. These modifications disrupt base pairing, altering the DNA double helix's structure. This destabilization can result in strand unfolding or breakage, leading to intracellular damage, loss of cellular functionality, and ultimately cell death.

These structural changes are detectable through UV-Vis spectroscopy, which highlights the absorption peak at 260 nm associated with the resonance of nucleotides. This technique is essential for studying strand alterations during oxidative events from a biophysical perspective.

As a second major target, we selected lipid vesicles, recognizing their importance as a model system for our study. These spherical structures, also known as liposomes or lipid bilayers, consist of single or multiple layers of phospholipids, and are well-studied in different fields including drug delivery, cellular studies, and membrane research. They are classified based on their size and lamellarity: Multilamellar Vesicles (MLVs) are characterized by several concentric lipid bilayers separated by aqueous compartments, while Small Unilamellar Vesicles (SUVs) feature only one lipid bilayer which encloses an aqueous core [214,215]. In this thesis, SUVs were selected due to their uniform size, high stability, and ability to simulate the structural and functional properties of biological membranes. By using SUVs, it was possible to simulate the lipid bilayers found in biological membranes, providing insights into how photosensitizing agents interact with and affect membrane integrity and function.

As third major component, proteins were not included in this study, as they had already been investigated previously by our research group, demonstrating that globular proteins such as bovine serum albumin (BSA) and myoglobin undergo unfolding and oxidative damage under blue light photoactivated mediated by N-TiO₂ treatment [83].

3.2 Experimental Results and Discussion

3.2.1 Spectroscopic and Morphological Properties of N-TiO₂ and AuNRs

The synthetic procedures used to prepare N-TiO₂ and AuNRs are presented in detail in Section 2.1.1 and 2.12. The materials' optical and morphological properties were then evaluated, followed by their characterization in terms of photodegradation activity using various model systems.

The investigation of the spectroscopic properties was crucial to assess whether nitrogen doping effectively modified the electronic configuration of TiO₂, particularly by narrowing the bandgap, which enhanced its photocatalytic performance under visible light exposure. Furthermore, the changes induced by the doping were also evident in its appearance, as the N-TiO₂ powder exhibited a yellow coloration, while the undoped TiO₂ was characterized by a white colour in accordance with literature [81,83]. Given its insolubility and tendency to precipitate in aqueous solution, its optical properties were assessed in solid powder form, through UV-Vis diffuse reflectance measurements with an integrating sphere [216,217].

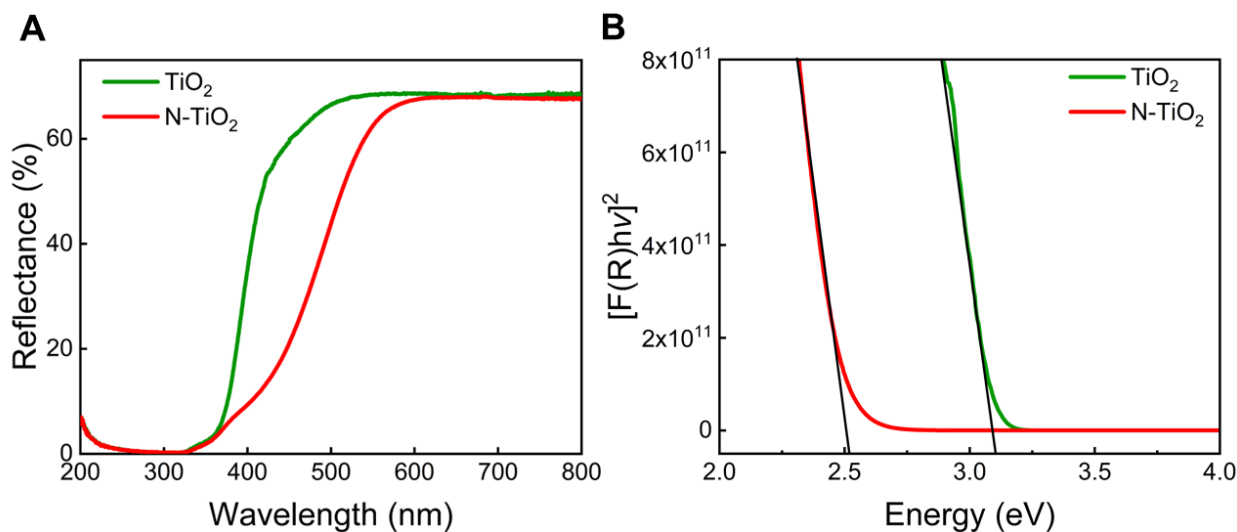


Figure 13: **Comparison of the optical properties of TiO₂ and N-TiO₂.** (A) Reflectance spectra as a function of wavelength, of TiO₂ (green) and N-TiO₂ (red) in the range 200-800 nm. (B) Tauc plots derived from reflectance data showing the relationship between $[F(R)hv]^2$ and photon energy (eV).

Notably, the spectral profile is significantly affected by nitrogen doping; specifically, the N-doped sample exhibits lower reflectance compared to the pristine one (Figure 13A). For N-TiO₂ sample, a significant increase in reflectance can be observed in the range above 350 nm, highlighting its increased ability to absorb light that is part of the visible spectrum. This difference is evident compared to pristine TiO₂, in a red shift of the reflectance, indicating that nitrogen has been effectively incorporated altering the electronic structure of N-TiO₂ and therefore the optical properties of the material.

Moreover, diffuse reflectance spectroscopy allows for the quantification of the bandgap energy, using the Kubelka-Munk model [218]. This method enables for the conversion of reflectance data into a form suitable for determining optical bandgaps. Figure 13B shows the Tauc plot of $(F(R)h\nu)^2$ against photon energy ($h\nu$), where $F(R)$ is the Kubelka-Munk function [219]. By analyzing the linear portion of the plot and extrapolating it to the energy axis, the bandgap energy can be determined. This analysis resulted in an approximate value of 3.1 eV for pristine TiO₂, while N-TiO₂ exhibited a reduced bandgap of around 2.5 eV, consistent with previously reported values [83]. This reduction in bandgap energy allows N-TiO₂ to absorb lower-energy visible light, as evidenced by the shift in the absorption spectra. Building on the analysis of the optical characteristics and bandgap narrowing of N-TiO₂, the investigation focuses on its morphological, compositional, and nanoscale characteristics.

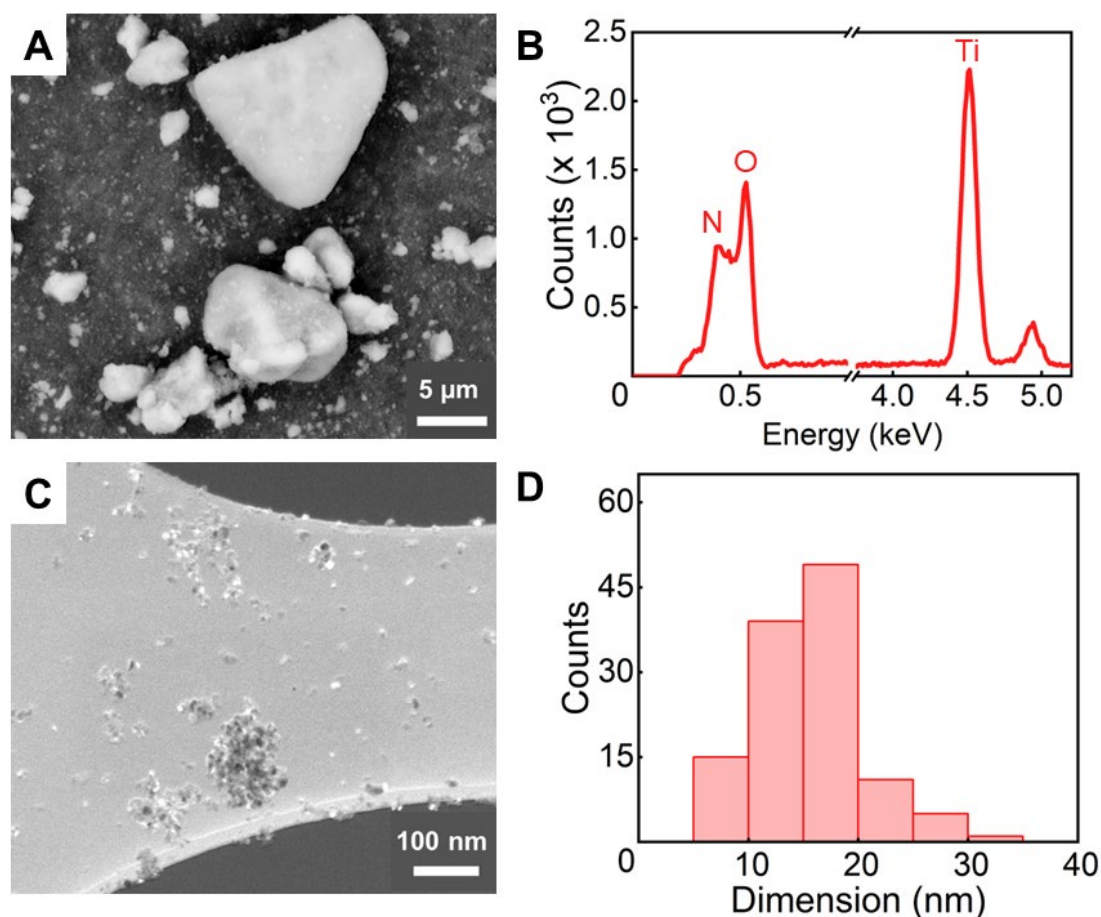


Figure 14: Representative Scanning Electron Microscopy (SEM) micrograph of N-TiO₂ (A), along with the corresponding Energy Dispersive X-ray (EDX) spectrum providing the elemental composition (B). Scanning Transmission Electron Microscopy (STEM) micrograph showing nanoscale structural details of N-TiO₂ (C). Histogram depicting the particle size distribution derived from analysis of the nanoscale fraction (D).

Figure 14A reveals heterogeneity in the particle morphology of N-TiO₂. The observed structures range from micrometer-sized aggregates to submicron particles.

The formation of these aggregates is well-documented in the literature, which identifies their occurrence as a drawback of the high-temperature calcination step typically employed as the final stage

of synthesis. During this process, particles undergo sintering, leading to the creation of larger structures [220]. As previously demonstrated, the sintering of individual particles during annealing is driven by the reduction of surface energy, which encourages coalescence [221]. Similarly, Teleki et al. observed that synthesized TiO_2 nanoparticles exhibit diverse sizes and morphologies, consisting of primary particles and aggregates [222].

Although the literature reports methods for isolating the nanometric fraction, such as ultrasonic treatment, high-pressure treatments, or the use of acidic solutions, in this study, we adopted a simplified approach focused on achieving greater efficiency and maximizing the yield from the synthesis. Indeed, isolating the nanometric fraction would have led to a drastic reduction in the overall product yield, resulting in unnecessary and potentially environmentally harmful waste. Given these considerations, we chose to assess the effectiveness of the material in its entirety. This decision is also supported by prior studies demonstrating the efficacy of the material in this heterogeneous form, consisting of a mixture of particles with different sizes [83]. Furthermore, the variability in the size of the nanostructured systems could prove beneficial for multiple applications beyond biomedical uses, such as including surface coatings using photocatalyst-based films or paints. For example, these materials could be used in the creation of self-sterilizing surfaces [223,224], or in environmental remediation efforts targeting pollutants [225]. In such applications, the larger particle sizes may offer different surface characteristics, enhancing various types of interactions and overall performance.

The elemental composition of the material, assessed through energy-dispersive X-ray spectroscopy (EDX) coupled with SEM, in Figure 14B, confirms that nitrogen atoms have been effectively integrated into the TiO_2 structure, in accordance with previous studies [81]. The distinct peaks corresponding to titanium (Ti), oxygen (O), and nitrogen (N) verifies that nitrogen doping has been successfully achieved.

At the nanoscale resolution, the structure of N- TiO_2 was observed using STEM, as shown in Figure 14C. The STEM micrograph reveals a dispersed fraction of nanometric particles. These nanoparticles are crucial in enhancing the material's optical and catalytic properties, as their small size increases the effective surface area available for light absorption and molecular interactions during photocatalytic processes. These observations align with measurements performed on the same sample by Ferrara et al. [83] using Atomic Force Microscopy (AFM), which similarly revealed comparable particle size distributions and morphological characteristics. The particle size distribution of the nanometric fraction is presented in the histogram in Figure 14D. The analysis reveals that the particle sizes are predominantly in the range of 10 to 30 nm, with most of the particles clustering around a mean size of approximately 20 nm.

Additionally, Z-potential measurements (data not shown) indicated a negative surface charge for the particles in aqueous solution, specifically measuring $-22.7 \text{ mV} \pm 5.2 \text{ mV}$. This parameter is essential for evaluating the particles' behaviour in suspension, as a sufficient surface charge prevents aggregation and promotes stabilization in solution, often achieved using acidic conditions.

After the characterization of N-TiO₂, the properties of AuNRs are here presented, as both materials are essential components of the final N-TiO₂/AuNRs composite. Following the synthesis procedure, the nanoparticles are dispersed in a colloidal solution stabilized by the cationic surfactant CTAB, allowing for absorption measurements to be performed in an aqueous medium [93,94,226]. The surfactant not only prevents aggregation of the nanoparticles but also facilitates the formation of a stable dispersion.

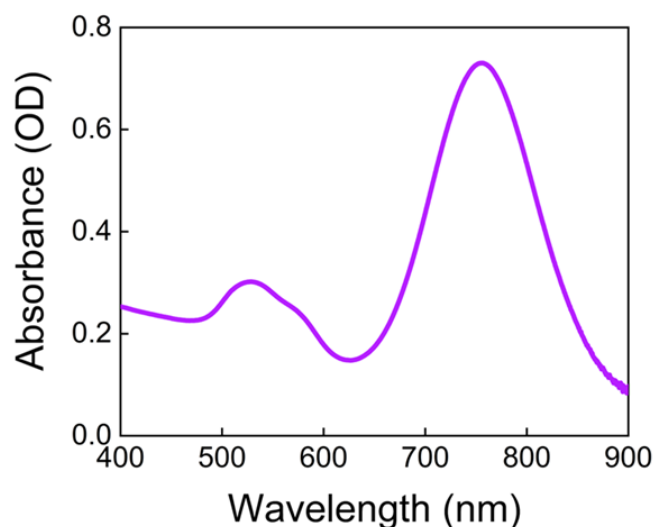


Figure 15: UV-Vis-NIR absorbance spectrum of gold nanorods in CTAB dispersion, showing optical density (OD) as a function of wavelength (nm) in the range of 400-900 nm.

In Figure 15 the absorption spectrum of AuNRs is shown, revealing the two main characteristic peaks: a strong absorption peak near 800 nm and a smaller peak in the visible range around 520–550 nm. The longitudinal peak, prominently observed at 750 nm, is named after the conduction electrons that oscillate along the longitudinal axis of the nanorods. This peak is highly dependent on the aspect ratio of the nanorods, with higher aspect ratios shifting the peak further into the NIR region [227]. The second peak in the visible region is attributed to electrons oscillating transversely along the minor axis of the nanorods [228,229].

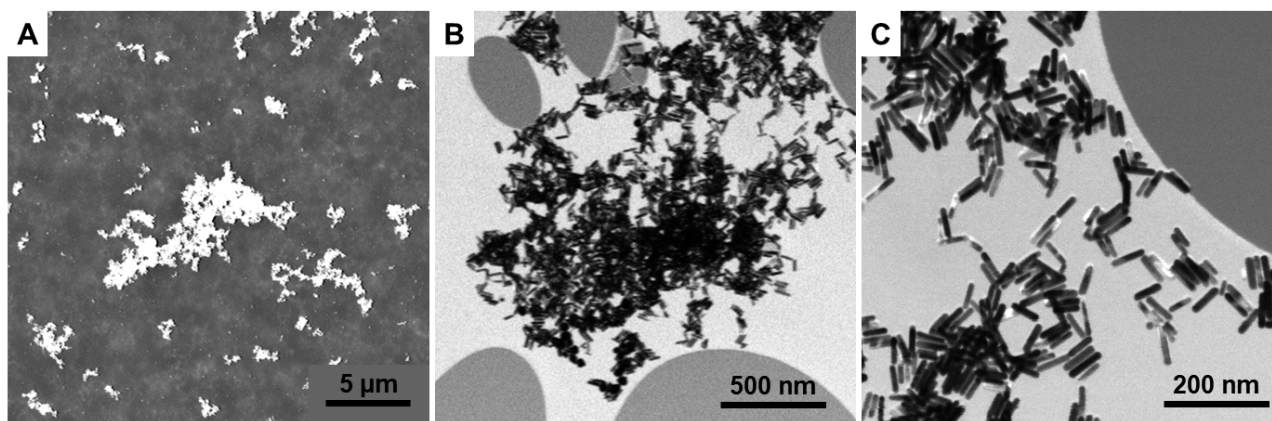


Figure 16: Morphological characterization of AuNRs in aqueous solution without CTAB stabilization: SEM micrograph (A); and STEM micrographs at two different magnifications (B, C).

The morphological characterization of the AuNRs was conducted in an aqueous solution without CTAB stabilization, reflecting the conditions under which they are incorporated into the N-TiO₂/AuNRs composite. Characterizing the AuNRs in their surfactant-free state was chosen to be aligned with their actual conditions in the N-TiO₂ composite. Without CTAB, the AuNRs are better positioned to interact directly with the photocatalyst, avoiding the insulating layer that the surfactant could introduce. Additionally, removing CTAB mitigates concerns over its poor biocompatibility, enhancing the composite's suitability for biological applications. Figure 16A shows an SEM micrograph illustrating the distribution of AuNRs aggregates deposited on a substrate. In the absence of CTAB, the AuNRs exhibit noticeable aggregation, driven by van der Waals forces, as expected for lyophobic colloids that naturally require stabilizing agents to prevent clustering. The lack of stabilization highlights the inherent instability of the AuNRs in aqueous solution without a surfactant. Figure 16 shows STEM micrographs at two different magnifications, allowing for higher resolution imaging of the AuNRs. In particular, Figure 16B shows clusters of AuNRs, while in Figure 16C it is possible to observe individual AuNRs with their characteristic rod-like morphology. High-resolution TEM micrographs further confirm the morphological findings from the SEM and STEM analyses.

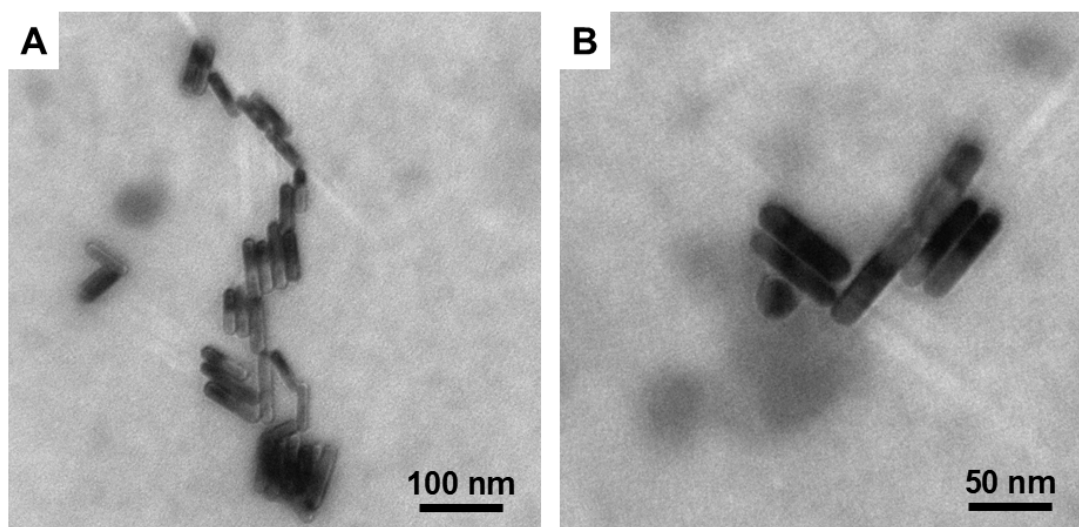


Figure 17: High-resolution TEM micrographs of AuNRs in aqueous solution without CTAB stabilization at two different magnifications (A, B).

Figure 17A shows the aggregation tendency of the AuNRs, illustrating their tendency to form loosely aligned structures in the absence of surfactant. This confirms the aggregation behaviour observed previously, where the nanorods tend to interact due to their lyophobic nature. Figure 17B, a higher magnification image reveals the uniform rod-like morphology of individual AuNRs, demonstrating their consistent size and aspect ratio. These TEM images offer a more detailed view of the AuNRs, highlighting their structural integrity and confirm the synthesis of nanorods with well-defined dimensions. Thanks to the high-resolution TEM analysis, which allowed for the measurement of the width and length of the AuNRs, crucial parameters for determining their aspect ratio, the size distributions are presented in Figure 18.

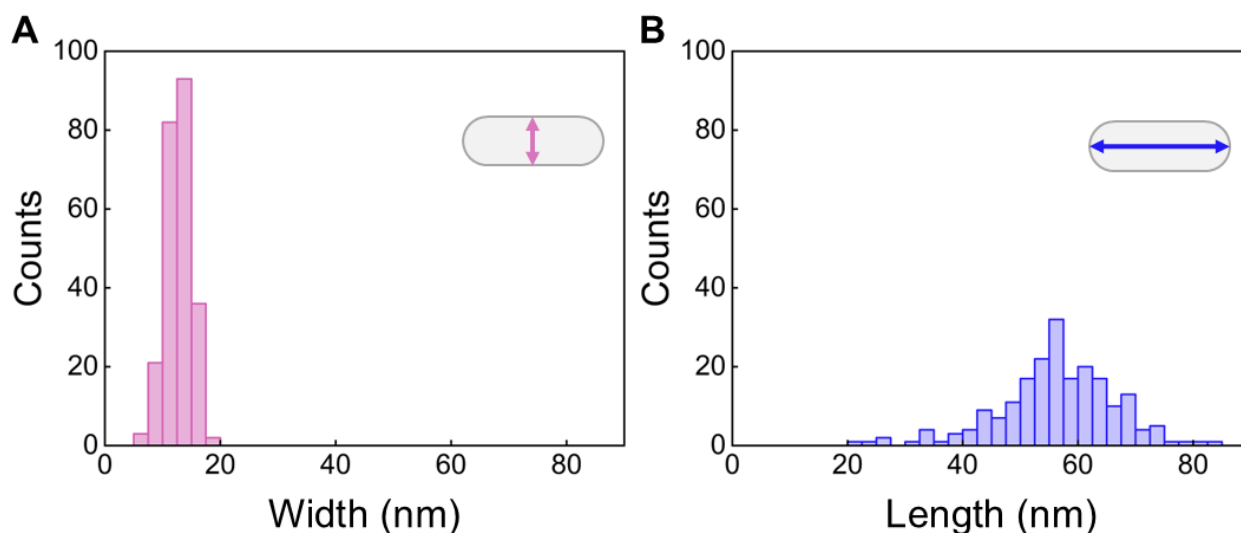


Figure 18: Histogram distribution of width (A) and length (B) of the AuNRs as measured from TEM images. Insets in both panels illustrate the corresponding measurement axes for width and length.

Figure 18A presents the distribution of the width of the AuNRs, which is found to be in the range between 10 and 25 nm, while Figure 18B shows the distribution of their length, which predominantly spans from 40 to 70 nm. These measurements confirm the rod-like morphology and narrow size distribution of the AuNRs.

The aspect ratio (R), defined as the ratio of the length to the width of the nanorods, is an important factor that influences their optical properties, particularly their plasmonic behaviour [90]. A higher aspect ratio causes the longitudinal plasmon resonance to shift toward longer wavelengths in the NIR region, which is important for applications. The aspect ratio of a dispersion of AuNRs can be evaluated using TEM or, alternatively, through UV-Vis spectroscopy analysis. Both methods provide comparable results, allowing for a reliable assessment of nanoparticle morphology. Notably, a well-established linear correlation exists linking the aspect ratio of AuNRs to the peak wavelength of the longitudinal plasmonic band ($\lambda_{\max}$) observed through UV-Vis spectroscopy [191,230]. The aspect ratio determined directly from the TEM measurements, yielded a value of 4.4 ± 1.6 . While the aspect ratio estimated from the absorption peak in the NIR region, which occurred at 754 nm, lead to an aspect ratio of 3.5 ± 0.5 . The aspect ratio values derived from both TEM and absorption measurements exhibit good consistency, with the differences falling within the measurement errors. This consistency further supports the validity of both methods for determining the aspect ratio and highlights their reliability in characterizing the AuNRs.

Furthermore, Dynamic Light Scattering (DLS) measurements were performed to assess the hydrodynamic size and dispersion of the AuNRs in solution (data not shown). The DLS results reveal a Z-average size of 73 ± 31 nm and a polydispersity index (PDI) of 0.67, which indicates a moderate degree of size variation within the sample. This result is consistent with the observed optical properties and further supports the notion that the synthesis and stabilization processes yield nanoparticles with controlled dimensions [93,190,231]. Additionally, the Z potential of the AuNRs was measured at 66.5 ± 10 mV, suggesting a stable colloidal dispersion due to the high positive charge on the nanoparticles, which promotes effective separation and prevents aggregation in solution.

3.2.2 Characterization of N-TiO₂/AuNRs composite

The N-TiO₂/AuNRs composite was thoroughly investigated to explore the interactions occurring between N-TiO₂ and AuNRs. As previously mentioned, the incorporation of AuNRs into N-TiO₂ was aimed at enhancing photocatalytic efficiency through the unique plasmonic properties of the AuNRs, which can facilitate visible light absorption and promote charge separation [232]. As discussed in Section 3.4, the composite was not synthesized through covalent bonding or complex surface modification strategies, but rather through a straightforward, minimalist approach. This method

involves the physical mixing of the two materials, resulting in a simple yet effective composite material. The goal was to produce a practical composite where the individual properties of N-TiO₂ and AuNRs are retained, thereby optimizing the performance of the final product without relying on intricate chemical interactions. The N-TiO₂, with its heterogeneity in particle size, was mixed with AuNRs following the removal of the CTAB surfactant, to allow optimal interaction at the surface between the two materials. This approach aligns with findings from other studies, which highlight the importance of removing surfactants such as CTAB to enhance the interaction between TiO₂ and AuNRs [233,234]. By eliminating residual CTAB, the surface of the materials becomes more accessible, facilitating optimal interaction and improving the efficiency of the composite material's photocatalytic properties under visible light conditions. The resulting composite, therefore, combines the photocatalytic properties of N-TiO₂ with the plasmonic properties of the AuNRs, leading to enhanced light absorption and charge separation.

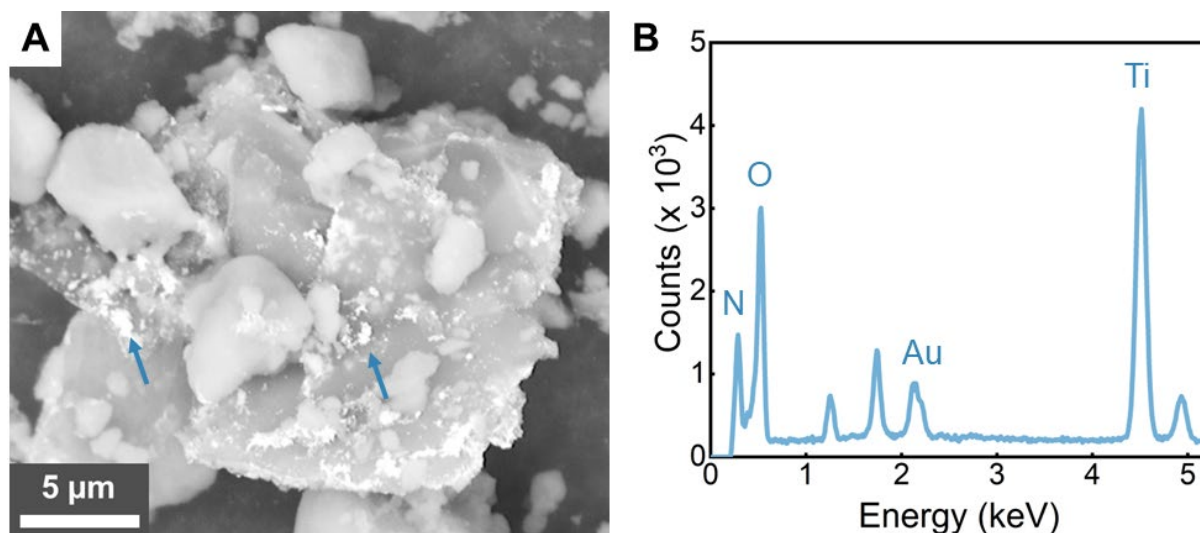


Figure 19: SEM micrograph of the N-TiO₂/AuNRs composite at the micrometric scale (A), with the corresponding EDX spectrum (B) obtained from the spots indicated by the arrows in the SEM micrograph.

Figure 19A shows a representative SEM micrograph of the N-TiO₂/AuNRs composite at the micrometric scale. The N-TiO₂ aggregates are clearly visible, with clusters of AuNRs adhering to their surface. Figure 19B presents the corresponding EDX spectrum, which confirms the presence of gold (Au) in the regions indicated by the arrows, in addition to the expected elements, such as nitrogen (N), oxygen (O), and titanium (Ti).

To further investigate the composite at the nanometric scale, STEM and TEM analyses were also performed, providing a more detailed visualization of the composite's structure.

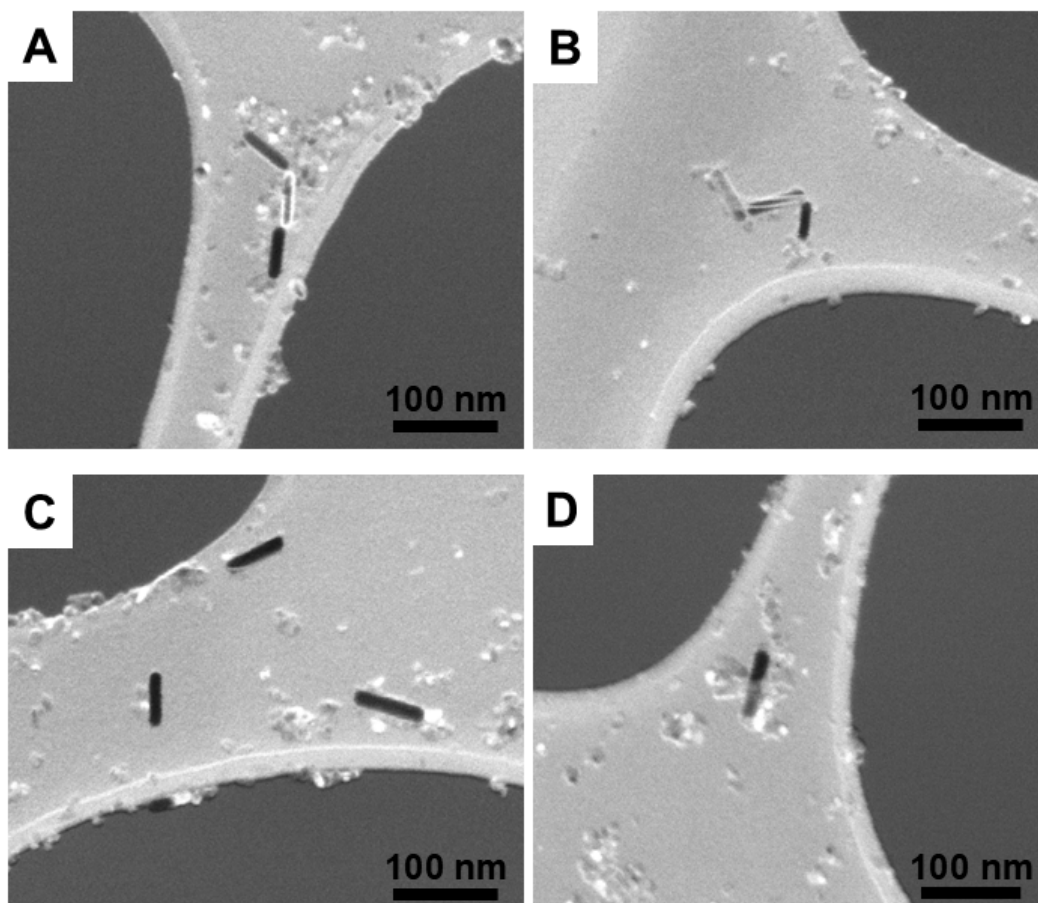


Figure 20: STEM micrographs of the N-TiO₂/AuNRs composite, showing different regions of the same sample. The light grey area in the images corresponds to the carbon mesh film on which the sample is deposited to allow optimal observation.

Figure 20A-D shows STEM micrographs of the composite, where individual AuNRs are visible among the N-TiO₂ particles. In comparison with the measurements performed on the bare AuNRs, in this case no aggregation or clustering is seen in the composite. This phenomenon is likely attributed to the electrostatic interactions, where N-TiO₂ facilitates the stabilization of AuNRs on its surface. As a result, the AuNRs remain well-dispersed throughout the composite, preventing the formation of large aggregates. This distribution could enhance the exposure of AuNRs to light and improve their interaction with the N-TiO₂ surface, thereby potentially boosting the composite's photocatalytic efficiency. The effective interaction of the two materials demonstrates the success of the physical mixing approach. Moreover, TEM imaging was also employed to investigate the interaction between AuNRs and N-TiO₂ at a higher resolution.

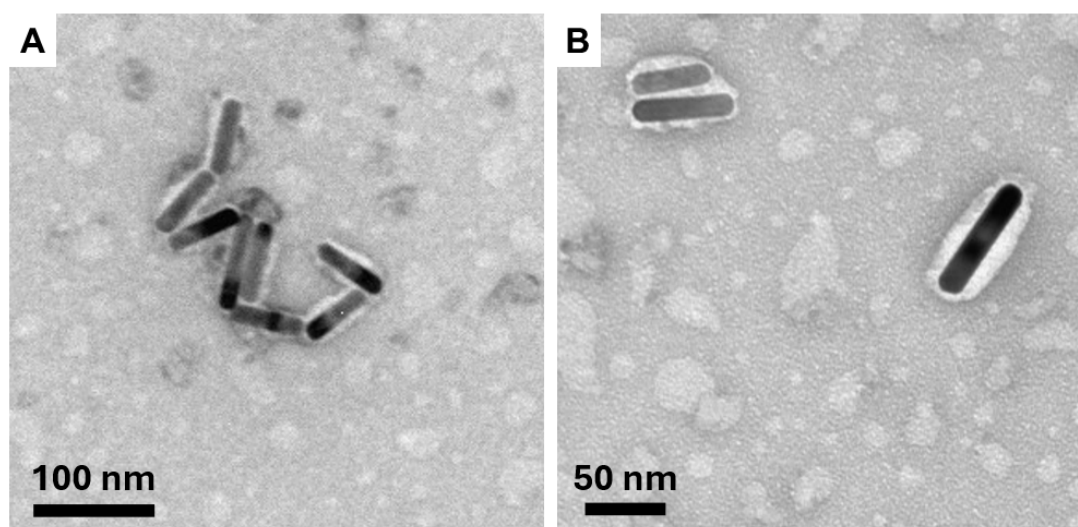


Figure 21: TEM micrographs of the N-TiO₂/AuNRs composite, at two different magnifications.

In Figure 21, TEM micrographs allow for the observation of a thin bright layer that appears to form around the surface of the AuNRs. This layer, which is not observed in bare AuNRs, likely originates from the deposition of the nanometric N-TiO₂ component, stabilizing the AuNRs and preventing aggregation. This observation aligns with findings by Jamil et al. (2022), who demonstrated that N-doped TiO₂ contributes to the stabilization of gold nanoparticles [235]. Moreover, numerous studies have demonstrated that TiO₂ coatings on AuNRs form a distinct layer around the nanorods, enhancing their stability and functionality. For instance, Fang et al. developed nanostructures composed of a gold core and a titania shell, which enhanced light absorption and promoted the production of ROS, highlighting the effective encapsulation of AuNRs by the titania shell [236].

Similarly, Ortega-Liebana et al. fabricated gold nanorods coated with titania, demonstrating an extended photocatalytic activity. These nanostructures functioned as enzyme-mimicking catalysts for glucose oxidation when exposed to near-infrared light [237].

These observations suggest that the N-TiO₂ matrix plays a crucial role in stabilizing the nanorods, as previously hypothesized. This stabilization is essential for maintaining the uniform distribution of AuNRs within the composite, ensuring optimal interaction between the two components.

3.2.2 Photocatalytic Degradation Efficiency of Methyl Orange

Photodegradation efficiency is a critical parameter in assessing the performance of photocatalytic systems in degrading target molecules when exposed to light. Methyl orange (MO), a synthetic azo dye frequently employed in various industrial applications, is chosen as the model compound for this study.

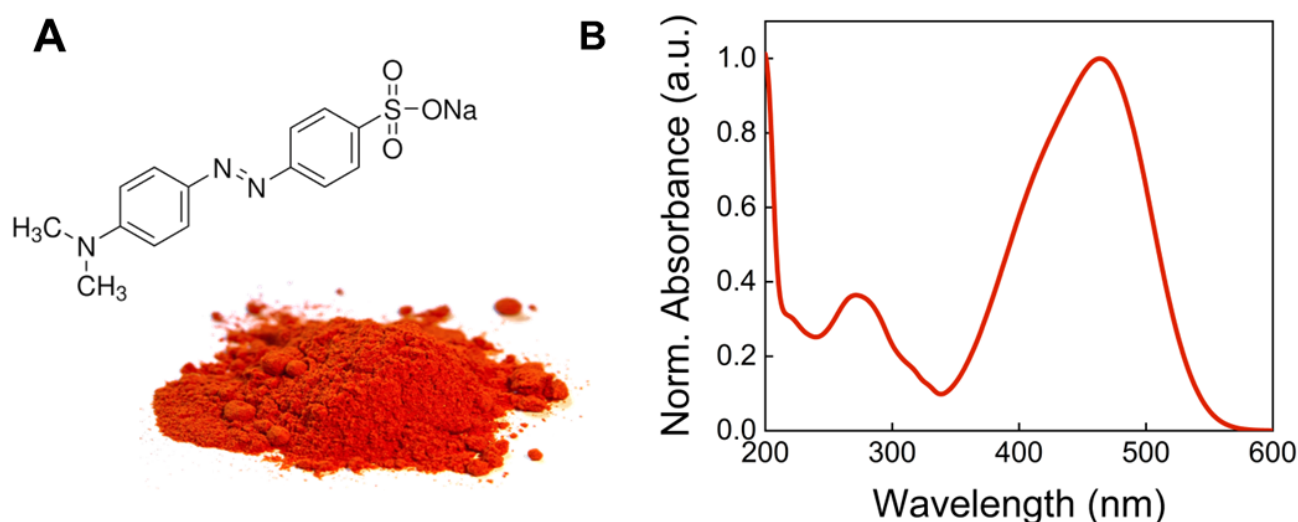


Figure 22: **Methylorange structure and absorbance spectrum.** (A) Molecular structure of MO (top) [238,239]. Image of MO in its solid form, appearing as a bright orange powder (bottom). (B) UV-Vis absorption spectrum of MO in the range 200-600 nm, normalized at the maximum peak absorption.

Its chemical structure (Figure 22A), characterized by the presence of an azo group (-N=N-) and sulfonate groups (-SO₃Na), which are essential for its optical properties. In its solid state, MO appears as an orange powder, which is typical of many azo dyes due to the conjugation of the azo group with the aromatic rings.

In Figure 22B presents the absorption spectrum of MO reveals its characteristic absorption peaks arising from its strong conjugated system: the azo chromophore absorbs prominently at 464 nm, influenced by the electron-donating dimethyl amino group, leading to absorption within the visible spectrum, that imparts the characteristic orange color to MO, while the π - π^* transition related to the aromatic rings appears at 270 nm, corresponding to absorption in the UV region [240,241]. Monitoring the degradation of MO through changes in its UV-Vis absorption spectrum allows for an assessment of the photocatalytic performance of the materials under investigation. Photocatalytic experiments were conducted using MO to investigate the degradation efficiency of N-TiO₂ alone and in combination with AuNRs in the composite material N-TiO₂/AuNRs, under visible light irradiation. Experiments were conducted using the biomedical LED device provided by the company *EmoLED*[®]. The spectrum of the blue LED is reported in the work by Ferrara et al. where it can be observed that it is centered at 420 nm [83]. Partner of this industrial PhD project, *EmoLED*[®] specializes in developing light devices for photobiomodulation to enhance wound healing. This light source was employed within the framework of this industrial PhD project to explore its potential in activating photocatalytic composites, with the aim of further advancing innovative biomedical applications.

Figure 23A illustrates the absorption spectra of MO samples collected at various time intervals during the photocatalysis experiments during the treatment with N-TiO₂ dispersion.

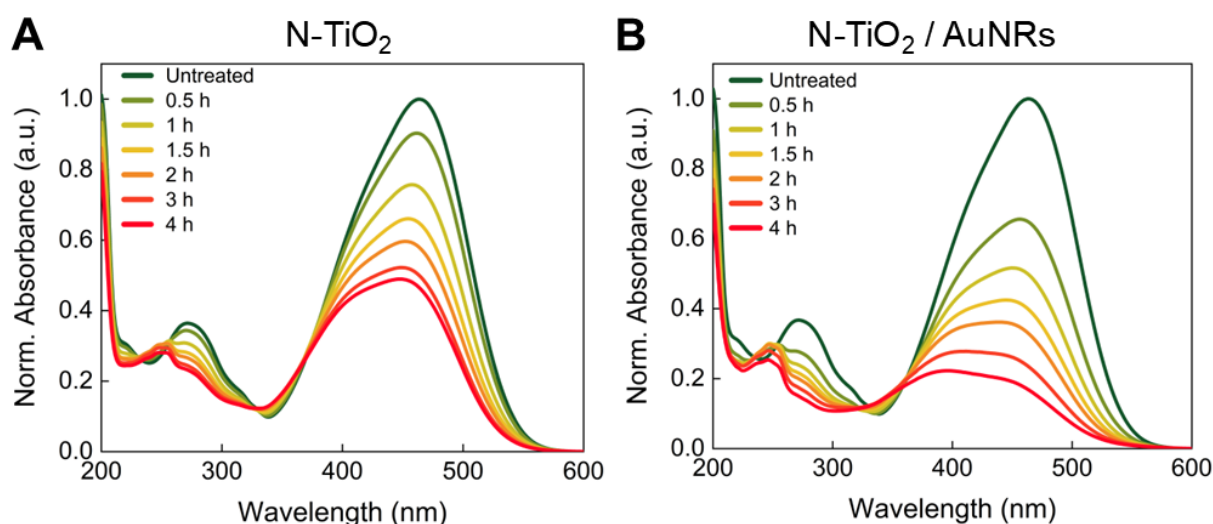


Figure 23: **Normalized UV-Vis absorbance spectra of MO during photodegradation** in the presence of N-TiO₂ (A) and N-TiO₂/AuNRs (B) under blue light LED irradiation. The spectra were recorded at different irradiation times, up to 4 hours, showing the evolution of absorbance in the 200–600 nm range. The 'untreated' spectrum refers to MO with the photocatalytic agent after incubation in the dark for 30 minutes. Data are normalized to the untreated sample's maximum absorption peak at 464 nm.

As the photocatalytic experiment progresses, significant modifications in the absorption spectra occur; likely due to the targeting of the electron-dense azo bond and the aromatic rings by the reactive oxygen species formed. The progressive reduction in the peak at 464 nm can be related to the photodegradation of MO into the gradual breakdown of the conjugated chromophore, particularly due to the cleavage of the azo bond [242,243]. This bond disruption results in a loss of conjugation between the rings, fragmenting the chromophore and causing a shift toward colorless or less intensely colored intermediates. The broadening of this peak over time further suggests the generation of a complex mixture of intermediate species with varied electronic structures, each contributing differently to the absorption spectrum. Moreover, in the UV region, notably at around 270 nm, is possible to observe the formation of smaller aromatic or aliphatic fragments as the MO molecule is broken down, forming less conjugated by-products, such as benzene derivatives or phenolic compounds, which exhibit absorption bands in this part of the spectrum. These changes suggest that photocatalytic degradation of MO proceeds through a series of oxidation steps, where the initial azo bond cleavage leads to further oxidation and ring-opening reactions, producing simpler molecular fragments [242,244,245]. These byproducts might include carboxylic acids, aldehydes, and other low-molecular-weight compounds that no longer contribute to visible light absorption but can affect the UV region.

These effects are further pronounced in the sample treated with the composite N-TiO₂/AuNRs, which demonstrates faster degradation due to an overall enhancement in the photodegradation process (Figure 23B). This improved degradation is attributed to the increased generation of ROS, facilitated by the synergistic effect of the N-TiO₂/AuNRs [195,246]. The higher stability and abundance of these

ROS allow for more efficient degradation of MO within the same irradiation time. Specifically, AuNRs help separate photogenerated electron-hole pairs through charge interactions, reducing recombination and promoting interfacial charge transfer [247].

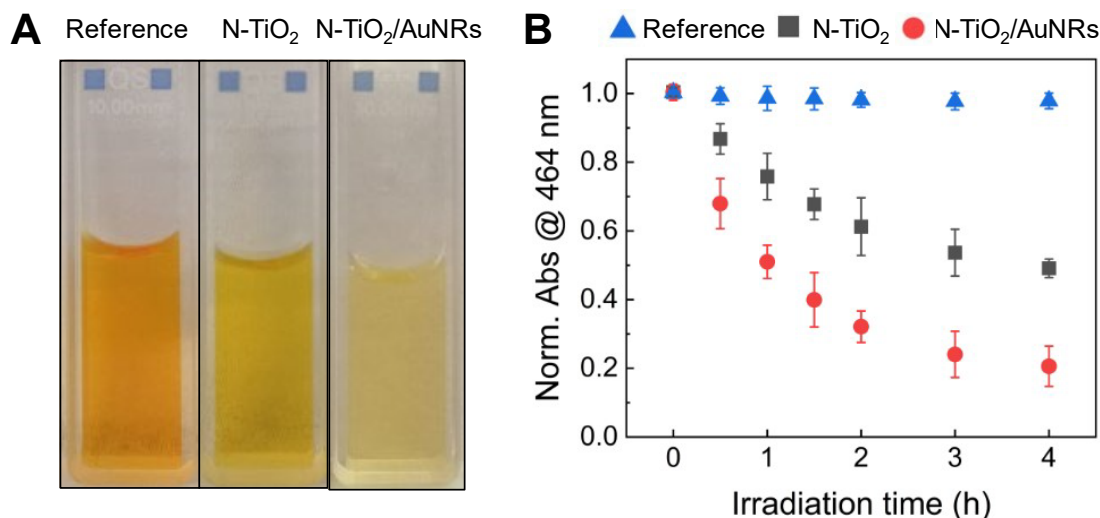


Figure 24: **Comparison of MO photodegradation under blue light LED irradiation after 4 hours:** images of the solutions after 4 hours of treatment: the reference sample, containing no photocatalytic material, and the solutions containing N-TiO₂ and N-TiO₂/AuNRs (A); normalized absorbance at 464 nm, plotted as a function of irradiation time for the reference, N-TiO₂, and N-TiO₂/AuNRs (B).

The visual evidence of photodegradation is illustrated by the color of the solutions in the cuvettes, as shown in Figure 24A. In comparison with the MO reference solution (left), which retains its typical orange color after being treated with light alone, the MO solution treated with N-TiO₂ (center) maintains a brighter yellow color. In contrast, the solution treated with the N-TiO₂/AuNRs (right) composite turns pale yellow after four hours of irradiation, indicating a more advanced state of degradation. This serves as further evidence of the degradation of the azo chromophore, which directly determines the macroscopic color change of the dye. To further highlight these changes, Figure 24B presents the maximum absorbance of MO as a function of irradiation time, showing that the presence of gold nanoparticles accelerates the photodegradation process, as observed from the shift in the main absorbance peak. Supporting this, Baiocchi et al. have shown through mass spectrometry analysis that MO degradation leads to the formation of various molecular fragments [245]. These fragments arise from the breaking of different molecular bonds, ultimately leading to the destruction of the chromophore, which explains the observed color change in the solutions.

3.2.4 Photocatalytic Degradation Efficiency of Calf-Thymus DNA

As detailed in section 3.1.3, CT-DNA serves as a valuable molecular model to study this type of reaction and its associated effects as it represents a well-known molecule for this type of studies. Specifically, using UV-Vis spectroscopy in the context of this study, enables for the detection of

conformational changes in the DNA double helix, providing insights into the mechanisms underlying oxidative photodegradation [248–250]. To this end, similarly to the experiments conducted with methyl orange, freshly prepared CT-DNA solutions were treated under identical conditions using the photocatalytic materials, and the absorption spectrum was recorded at various time intervals.

We evaluated the effect of the oxidative process triggered by N-TiO₂ on CT-DNA upon the same blue light irradiation. This treatment can indeed damage the DNA double helix, and such changes can be monitored by analyzing the absorption spectrum.

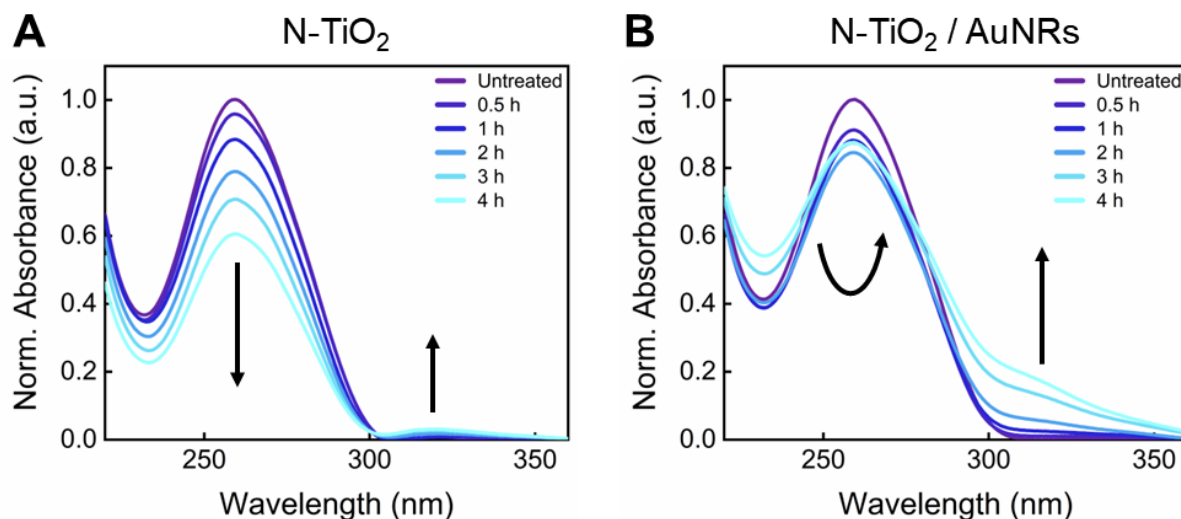


Figure 25: Normalized UV-Vis absorption spectra of CT-DNA treated with N-TiO₂ (A) and N-TiO₂/AuNRs (B) and exposed to blue light irradiation for up to 4 hours. The spectra are normalized to the maximum absorbance of the untreated sample. Arrows indicate the main spectral modifications occurring during the treatment.

Figure 25A demonstrates a progressive reduction in the absorption peak at 260 nm with increasing irradiation time. Additionally, a slight increase in absorption is observed in the region beyond 300 nm, where native DNA typically does not absorb. Moreover, these spectral changes suggest conformational alterations in the DNA double helix, which can potentially be linked to oxidative modifications of the nucleotide bases. Spectral modifications are highlighted by the arrows, showing a hypochromic change observed at 260 nm reflect structural rearrangements or disruptions of the double helix, which reduce the accessibility of the DNA chromophore and, consequently, lead to a progressive decrease in absorption intensity [251–253]. Moreover, a minor rise in absorption can also be detected beyond 300 nm, which may correspond to the presence of oxidized nucleobases, such as 8-oxoguanosine. This observation may reinforce the hypothesis that the observed conformational alterations are associated with oxidative modifications of nucleotide bases, possibly mediated by N-TiO₂ under blue light irradiation [254].

To further investigate the oxidative processes triggered by photocatalysts on CT-DNA, we analyzed the potential changes induced by treatment with the composite N-TiO₂/AuNRs, to assess whether this

composite is more effective in photodegrading the target molecule. In this case, the spectral changes are more complex, as shown in Figure 25B. Initially, a hypochromic effect is observed, indicating the progressive deformation of the DNA double helix as oxidation proceeds. However, after two hours of irradiation, an increase in absorbance at 260 nm becomes evident, corresponding to a hyperchromic effect. This phenomenon is well known to indicate the unfolding of the DNA double helix, caused by the oxidation of nucleotide bases [212,255,256]. Oxidative modifications disrupt the uniform stacking interactions required to stabilize the helical structure. As the bases become more exposed to the solvent, the absorbance at 260 nm increases.

Furthermore, a significant rise in absorbance around 320 nm is observed. While this feature was previously noted for N-TiO₂ alone, the magnitude of this increase is much more pronounced with the N-TiO₂/AuNRs composite. This pronounced spectral change suggests the accumulation of oxidized nucleotide bases, which absorb in this spectral region [256,257]. In this context, guanine, due to its low ionization potential, is particularly susceptible to oxidation, reacting with singlet oxygen and hydroxyl radicals ($\bullet$ OH) generated during photocatalysis. Its oxidized form, 8-oxo-guanine, absorbs above 300 nm, contributing to the spectral changes observed. This aligns with the reported increase in absorbance around 320 nm, further supporting the hypothesis of guanine oxidation as a key event during DNA structural alteration under oxidative stress [210,258,259].

These more extensive oxidative modifications are attributed once again to the enhanced photocatalytic properties of the N-TiO₂/AuNRs composite, which arises from the stabilization of ROS mediated by the AuNRs, which enhance electron transport and extend the charge carriers' lifetime, thereby amplifying the oxidative effects on the DNA. This highlights the superior efficiency of the composite system in propagating oxidation and inducing structural alterations in the DNA.

3.2.5 Photocatalytic Degradation of Calf-Thymus DNA within a Chitosan Hydrogel Matrix

To develop biomaterials that integrate the antibacterial properties of photocatalytic agents, we designed a chitosan-based hydrogel capable of encapsulating N-TiO₂ and the N-TiO₂/AuNRs composite. This system serves as a potential delivery platform for localized applications against bacterial infections or biofilms, such as topical treatment of chronic infected wounds. The hydrogel provides controlled coverage of the targeted area, which can be irradiated on demand, enabling advanced antibacterial treatment. Additionally, the hydrogel can be easily removed after therapy, offering a versatile and efficient approach. This strategy ensures precise dosing and stable formulation of photocatalytic materials, presenting promising therapeutic benefits [83,260,261].

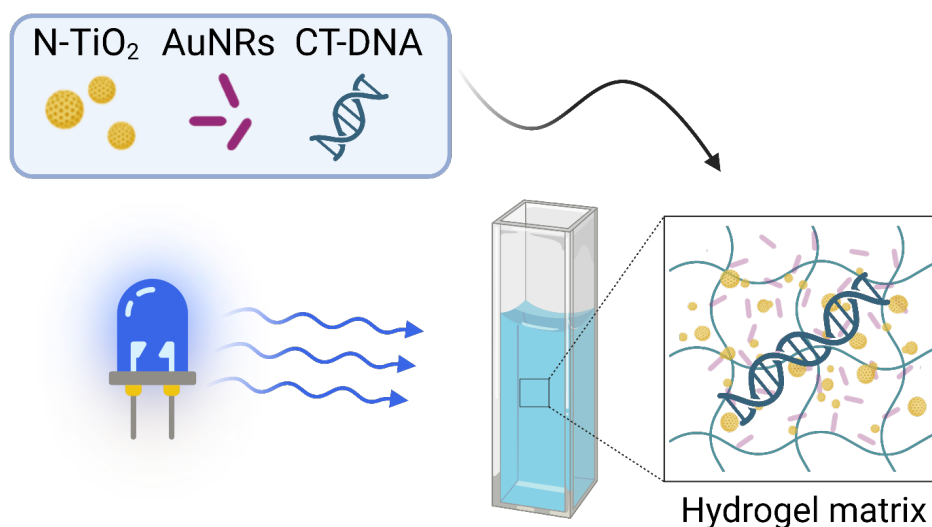


Figure 26: **Experimental setup for CT-DNA degradation within a chitosan hydrogel matrix using N-TiO₂/AuNRs under blue LED light irradiation.** The photocatalyst (N-TiO₂/AuNRs) is embedded within the chitosan hydrogel matrix, which was swelled in a solution of CT-DNA. The sample is irradiated with 420 nm blue light, and the spectral changes over time are monitored by absorbance spectroscopy. Created with Biorender.com

This matrix, illustrated in Figure 26, offers several advantages for the objectives of our study. By incorporating target molecules such as DNA into the hydrogel, the system provides not only an optically transparent medium but also a biomimetic matrix that closely resembles a native biological environment. Its high hydration maintains the native-like conformation of DNA while facilitating oxidative processes. Our approach was based on the use this matrix, as it had previously been shown to be effective in studying the oxidative effects on globular proteins [83].

Furthermore, immobilizing the components within the hydrogel mitigates common issues encountered in colloidal suspensions, such as sedimentation and aggregation, enhancing experimental reliability. This immobilization also eliminates the need for repeated sample retrieval and processing at each time point. Consequently, the approach allows for continuous spectroscopic monitoring of the reaction progress, significantly improving the efficiency and accuracy of the experimental workflow.

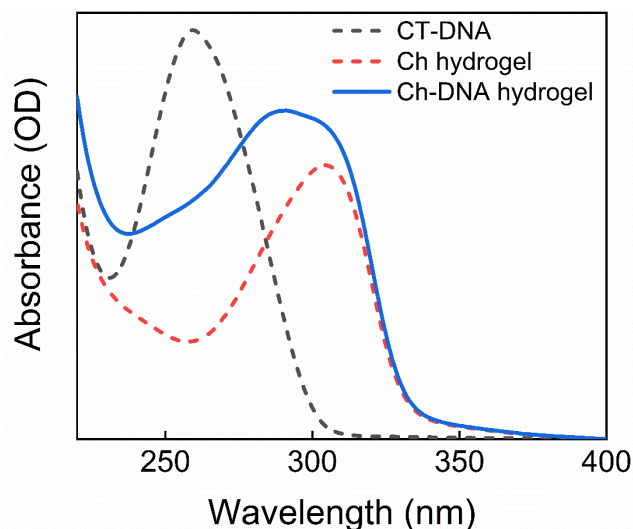


Figure 27: **Normalized UV-Vis absorption spectra** of CT-DNA in solution (black dashed line), the chitosan/EPEG hydrogel swollen in water (red dashed line), and the chitosan/EPEG hydrogel swollen in a CT-DNA solution (blue solid line). Spectra were normalized at their maximum.

Figure 27 demonstrates the optical behaviour of the chitosan hydrogel system. The chitosan hydrogel swollen in water exhibits a broad absorption band around 305 nm, arising from the intrinsic properties of the polymer matrix. When CT-DNA is incorporated into the hydrogel, its characteristic absorption is retained, though slightly broadened. This indicates interactions between the DNA and the hydrogel matrix, likely mediated by electrostatic attractions, resulting from the negative charge of its phosphate backbone and the positive charge of chitosan, in addition to potential hydrogen bond formation [262,263]. These interactions slightly alter the DNA spectral features while preserving its overall structural characteristics.

This system provides an effective model for studying DNA oxidative modifications. By immobilizing DNA and photocatalytic agents within the hydrogel, the matrix minimizes issues such as sedimentation and aggregation, enabling continuous monitoring of structural changes under photocatalytic conditions [225]. The hydrophilic nature of the gel also supports a native-like biomolecular conformation, ensuring the reliability of spectroscopic observations [264].

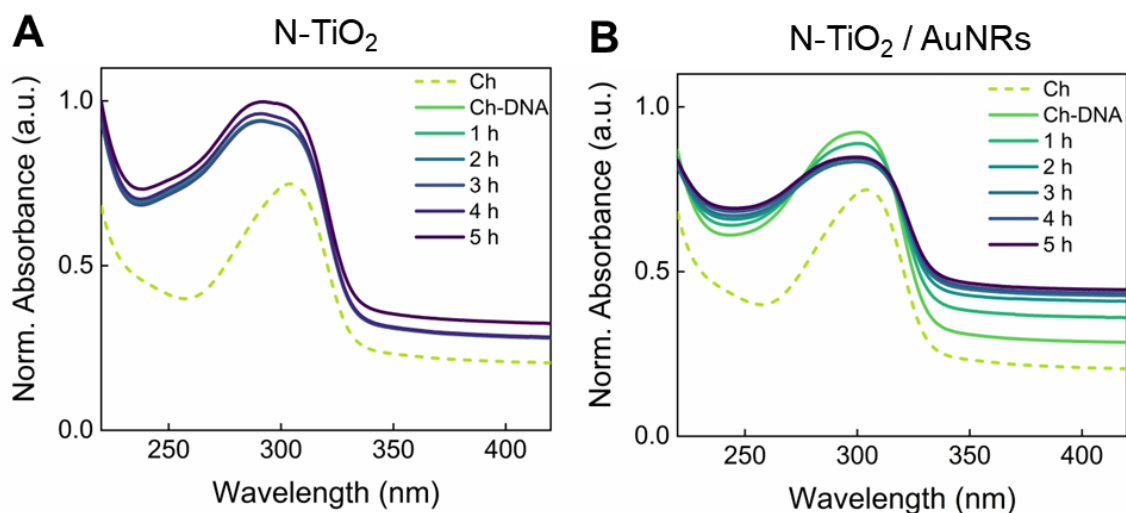


Figure 28: **Normalized absorbance spectra of chitosan hydrogel matrixes** treated with N-TiO₂ (A) and N-TiO₂/AuNRs(B) under various experimental conditions. The dotted line represents the chitosan gel swollen in water combined with N-TiO₂ or N-TiO₂/AuNRs (Ch), while the solid cyan line corresponds to the chitosan gel swollen in the DNA solution combined with N-TiO₂ or N-TiO₂/AuNRs (Ch-DNA). The remaining curves illustrate the temporal evolution of the Ch-DNA gel during visible blue light irradiation, with spectra recorded at 1, 2, 3, 4, and 5 hours. All spectra are normalized to the maximum absorbance of the sample treated for 5 hours with N-TiO₂.

It is observed that in the case of the hydrogel matrix composed of chitosan and embedded with DNA, treated solely with N-TiO₂, the detectable changes attributable to DNA oxidation remain relatively limited (Figure 28A). Over time, a gradual enhancement in absorption is detected around the 260 nm region, indicating minor oxidative modifications in the double helix structure. Interestingly, the behaviour of the sample treated with N-TiO₂/AuNRs resembles closely what is observed in solution (Figure 28B). In this case, the absorption at 260 nm initially decreases, likely due to structural rearrangements or compaction of the DNA induced by the nanoparticles. After the first two hours, a subsequent increase in absorption is observed, which may suggest progressive unfolding of the double helix.

In both treatment conditions, turbidity increases around the 400 nm region, particularly in the sample treated with N-TiO₂/AuNRs. This turbidity is likely caused by structural modifications in the chitosan hydrogel matrix, including dehydration, polymer reorganization, and localized temperatures increases.

3.2.5 Evaluation of Photocatalytic Effects on Model Lipid Membranes Using Fluorescent Probes for Lipid Peroxidation and Membrane Order

Lipid peroxidation represents a key phenomenon in understanding the impact of oxidative stress on biological membranes, with relevance to antibacterial strategies [265,266]. Liposomes serve as an ideal model system for such studies, allowing controlled experimental conditions to mimic the structural and functional dynamics of cellular membranes, as discussed in Section 3.1.3.

Building on this foundation, the following discussion examines oxidative damage to lipid membranes

induced by photocatalytic processes. To this end, SUVs were employed due to their well-defined structural properties and relevance as models for biological membranes [267,268]. These vesicles were formed using a combination of saturated lipids 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (POPG), which closely mimic bacterial membrane lipids due to their zwitterionic and anionic properties, respectively. Unsaturated lipid 1-palmitoyl-2-arachidonoyl-sn-phosphatidylcholine (PAPC) was included to represent a model of oxidatively sensitive lipids commonly found in membranes across various biological systems and frequently used in studies of this kind due to its established role in lipid peroxidation research [269–271]. Together, POPC, POPG, and PAPC are considered gold standards in lipid peroxidation experiments for their ability to provide a robust and physiologically relevant platform [272].

In order to assess the effects of oxidative treatments on the vesicular membranes, fluorescence studies were conducted using two widely recognized fluorescent probes, C-11 BODIPY^{581/591} and Laurdan, which are detailed in Section 2.5. C-11 BODIPY^{581/591} is used to monitor membrane fluidity and phase transitions, as it integrates into the lipid bilayer and provides insight into membrane oxidation rates, particularly it detects oxidative damage to the membranes by monitoring its own oxidation, which serves as an indicator of membrane oxidation. From this point onward, we will refer to this probe simply as BODIPY. Laurdan, a probe sensitive to hydration and phase behaviour, offers complementary information on lipid bilayer structure and dynamics. By labeling the vesicles with these probes, we were able to evaluate how their membranes responded to treatments with the photocatalysts studied in this chapter.

For a better evaluation of the effects of BODIPY, we first present a representative fluorescence spectrum as a comparison for freshly prepared vesicles labeled with the probe. A characteristic fluorescence is observed with three main peaks at 594, 545, and 516 nm. Notably, the peak at 594 nm is the characteristic red emission of this probe, which is also the peak that disappears once the vesicles are treated with photocatalytic material under light irradiation for 6 hours. As a result of oxidation, a shift in emission occurs, with the red peak at 594 nm disappearing and the green emission peak at 520 nm becoming dominant [142,270,273]. The chemical modifications that lead to these spectral changes have been discussed in detail in Section 2.5.1. Briefly, these modifications involve changes in the conjugated aromatic system due to the targeting of the diene by ROS, which disrupt the conjugation and result in the observed shift in fluorescence. This change in the emission spectrum reflects the oxidation of the BODIPY probe and provides direct evidence of the oxidative effects on the vesicular membranes [140].

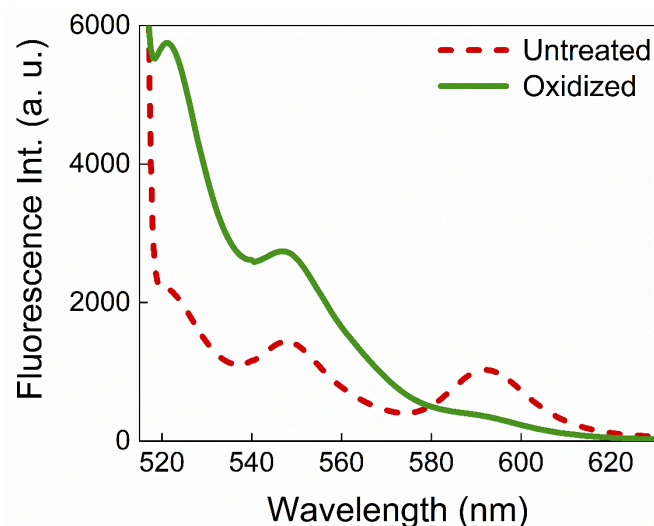


Figure 29: **Representative fluorescence emission spectra of BODIPY-labeled SUVs in aqueous solution**, under excitation at $\lambda_{\text{exc}} = 485$ nm. The red dashed line represents the spectrum of untreated vesicles, while the green solid line corresponds to the spectrum of vesicles treated with photocatalytic material under light irradiation for 6 hours, showing the oxidation-induced shift in fluorescence emission.

As shown in Figure 29, the untreated vesicles exhibit a strong fluorescence intensity at 594 nm (red dashed line), corresponding to the typical red emission of the BODIPY probe. After six hours of treatment with photocatalytic material under light irradiation, the emission spectrum shifts, and the fluorescence intensity at 594 nm diminishes, while the intensity at 520 nm increases, indicating the oxidation of the probe (green solid line).

The incorporation of this probe enables the temporal monitoring of the process, allowing kinetic analyses during irradiation with visible light to assess what is happening to the probe and, consequently, to the vesicles as a result of the treatments. Specifically, this approach facilitates the mapping of these changes, revealing characteristic trends over time. Before discussing the differences between the treatment with N-TiO₂ and the N-TiO₂/AuNRs composite, it is worth exploring the mechanism behind the changes in the fluorescence emission spectra of BODIPY upon oxidation. To this end, a fluorescence intensity contour plot illustrates the time-dependent oxidation of the vesicles, as indicated by the changes in the characteristics peak of the BODIPY probe (Figure 30).

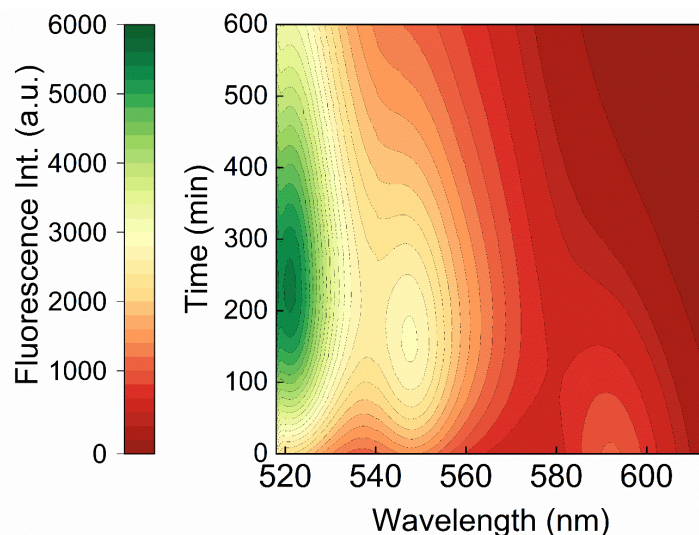


Figure 30: **Fluorescence intensity contour plot of vesicles labeled with BODIPY**, excited at $\lambda_{exc} = 485$ nm, over time during photocatalytic treatment with $N\text{-TiO}_2/\text{AuNRs}$ under visible light irradiation. The x-axis represents emission wavelength (520–600 nm), and the y-axis represents time (0–600 minutes). The color scale indicates fluorescence intensity (a.u.) over time, revealing changes in the distinctive peaks of the probe.

Initially, at early time points, it is possible to note a strong red emission peak around 594 nm, typical of the unoxidized probe. Over the course of the treatment, a clear decrease in intensity at 594 nm is observed, accompanied by an increase in the intensity around 520 nm, which reflects the formation of the oxidized probe. The gradual shift in emission with increasing time highlights the kinetics of the oxidative damage to the membranes. As previously mentioned, the characteristic strong red emission peak at 594 nm can be observed at early time points, corresponding to the unoxidized probe. As oxidation progresses, the intensity at 594 nm decreases, while the intensity around 520 nm increases, indicating the formation of the oxidized probe.

Literature provides various approaches to evaluate oxidative damage, including monitoring the decay of the 594 nm peak, which correlates with the breakdown of the diene conjugate and the loss of the phenyl moiety [142,146]. Some studies also utilize the polarization index (PI), calculated from the intensity ratio of the two emission peaks (520 nm and 594 nm) [269,272]. However, these methods often oversimplify the behaviour of BODIPY due to the complex nature of the by-products formed during oxidation.

Therefore, it is crucial to account for the dye's fluorescence characteristics within this framework. As oxidation progresses, reactions may form products with an enhanced green emission peak at 520 nm, but these species can later degrade into non-fluorescent forms (quenching), which may continue to undergo oxidation without being detectable. This could explain the initial increase in fluorescence at 520 nm, followed by a decrease as oxidation advances [274,275].

Additionally, excimer formation has been observed for different BODIPY dyes with various functional groups, depending on the solvent and local dye concentration. Excimers are formed from tightly

packed unoxidized dye molecules and they emit at a longer wavelength, around 590 nm. As oxidation proceeds, the dye becomes unavailable for excimer formation, leading to a decrease in red fluorescence at 594 nm [146]. Simultaneously, the green fluorescence at 520 nm increases initially due to reduced excimer formation and later decreases as more dye molecules become fully oxidized. This decrease in green fluorescence may be due to quenching, as previously mentioned.

Therefore, the red emission at 594 nm is a more reliable indicator of oxidation rates, as the decrease in the 594 nm peak (excimer peak) is closely related to the amount of unoxidized probe remaining in the system.

To evaluate whether excimer formation occurs under our experimental conditions, we studied different concentrations of the BODIPY probe in the as-studied lipid vesicles system. Figure 31 shows the fluorescence spectra at different concentrations, ranging from 233 nM to 933 nM. As the probe concentration rises, a corresponding increase in the emission peak intensity at 594 nm is detected, indicating excimer formation. This trend supports the hypothesis that excimers form more readily at higher concentrations, as the increased concentration of BODIPY molecules promotes their interaction and subsequent aggregation, leading to the characteristic red shift in the emission spectrum.

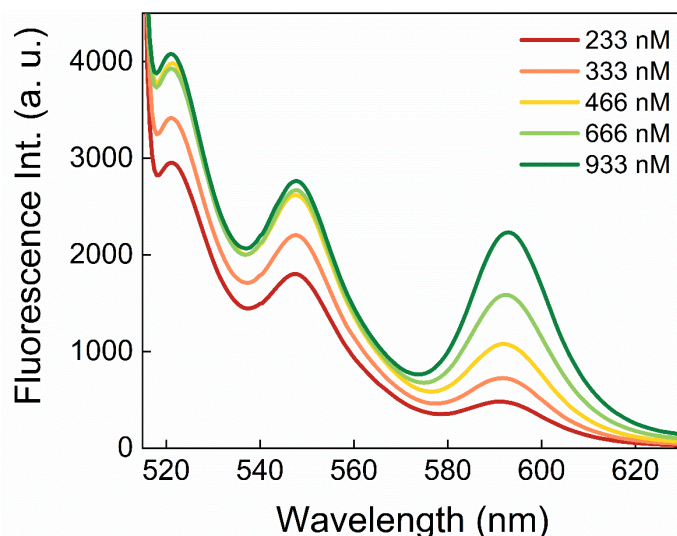


Figure 31: **Fluorescence emission spectra of the BODIPY probe** excited at $\lambda_{exc} = 485$ nm, incorporated into vesicles dispersed in buffer, without photocatalytic materials, after 2 hours of incubation to reach equilibrium. The dye was incorporated at different concentrations (233 nM, 333 nM, 466 nM, 666 nM, and 933 nM).

In our experiments, we used a concentration of 466 nM, which, based on the spectral data, appears to be within the range where excimer formation is significant. This concentration allowed us to study the effects of oxidation and excimer behaviour effectively without reaching a saturation point where the excimer signal would dominate excessively [145,147,276].

Building on these observations, photocatalytic oxidation experiments were conducted using vesicles labeled with BODIPY to investigate the emission behaviour in both the green and red channels. The

aim was to evaluate whether, similarly to the cases of methyl orange and DNA, the combination of AuNRs within the composite improves its photocatalytic efficiency. The temporal behaviour of fluorescence at 520 nm, corresponding to the green channel, was analyzed to investigate the oxidation dynamics in the vesicles. The results reveal distinct fluorescence patterns, reflecting the extent of oxidation under different conditions.

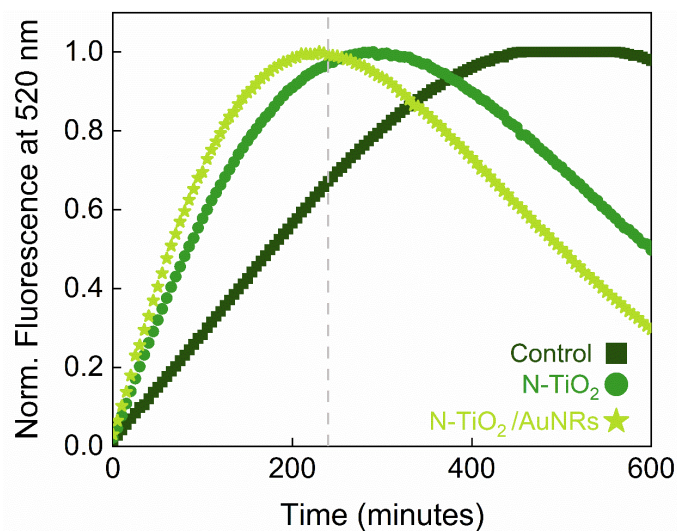


Figure 32: **Fluorescence emission at 520 nm as a function of time (0-600 minutes)**, normalized to its maximum, for BODIPY-labeled vesicles treated under visible light irradiation. Spectra were recorded every 5 minutes, with excitation at $\lambda_{exc} = 485$ nm. The three conditions are represented: control (dark green squares), N-TiO₂ (green circles), and N-TiO₂/AuNRs (light green stars). The dashed vertical line at 240 minutes indicates the 4-hour time point, used for comparison with prior photocatalytic experiments.

Specifically, the experiment underscores the difference in photocatalytic efficiency between N-TiO₂ and N-TiO₂/AuNRs composites (Figure 32). The N-TiO₂/AuNRs composite generates a faster increase in fluorescence, suggesting enhanced oxidation activity compared to N-TiO₂ alone. Accordingly, the point at which the green fluorescence begins to decrease occurs earlier compared to N-TiO₂, suggesting that the presence of AuNRs might not only accelerate the rate of oxidation but could also facilitate a more efficient oxidative process. This early decrease in fluorescence could be attributed to a faster depletion of the probe or the formation of oxidized species, which directly affect the fluorescence signal as before discussed. This supports the hypothesis that incorporating AuNRs into the N-TiO₂ composite improves oxidative processes, resulting in a more significant modulation of fluorescence. The dashed vertical line at 240 minutes, marking the 4-hour time point used in prior photocatalytic experiments for MO and DNA, is used as a reference. This facilitates the evaluation of the consistency of the observed effects and provides a foundation for comparing the vesicle system with other experimental setups.

Following this analysis, similar patterns are observed in the fluorescence behaviour at 594 nm (red

channel). As with the green channel, the data highlight distinct differences in photocatalytic efficiency between the N-TiO₂ and N-TiO₂/AuNRs composites (Figure 33).

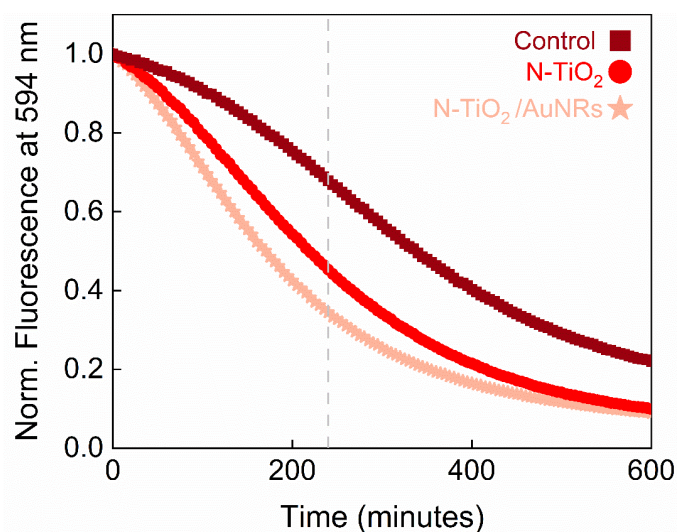


Figure 33: **Fluorescence emission at 594 nm as a function of time** (0-600 minutes), normalized to its maximum, for BODIPY-labeled vesicles treated under visible light irradiation. Spectra were recorded every 5 minutes, with excitation at $\lambda_{exc} = 485$ nm. The three conditions are represented: control (dark red squares), N-TiO₂ (red circles), and N-TiO₂/AuNRs (light red stars). The dashed vertical line at 240 minutes indicates the 4-hour time point, used for comparison with prior photocatalytic experiments.

The N-TiO₂/AuNRs composite exhibits a faster decrease in fluorescence compared to the N-TiO₂-only condition, suggesting that the presence of AuNRs accelerates the photocatalytic oxidation process. Specifically, the faster decline in fluorescence indicates that the BODIPY probe is being oxidized more quickly in the presence of the N-TiO₂/AuNRs composite, likely due to the enhanced photocatalytic activity provided by the AuNRs. The incorporation of AuNRs into the N-TiO₂ composite seems to facilitate a more efficient oxidation mechanism, leading to a quicker depletion of the unoxidized probe and a faster disruption of excimer formation, which directly reduces fluorescence at 594 nm.

The different rates in fluorescence emission changes at both 520 nm (green channel) and 594 nm (red channel) in the sample treated with N-TiO₂/AuNRs compared to the sample treated with N-TiO₂, reinforces the hypothesis that the AuNRs significantly contribute to the enhancement of the oxidative reactions. As a result, the N-TiO₂/AuNRs composite not only accelerates oxidation but likely improves the overall efficiency of the photocatalytic reaction, resulting in a more rapid breakdown of the probe. However, even in the control condition (vesicles with no photocatalytic treatment), fluorescence reduction is observed, albeit more slowly. This is likely due to the oxidation of the BODIPY probe by oxygen present in the buffer and the surrounding environment, although the process is much less efficient without the photocatalytic materials. This slower decrease in fluorescence in the control reflects the limited oxidation that occurs in the absence of photocatalytic treatment which, however, despite the experimental measures, cannot be completely eliminated [269,270].

In addition to the BODIPY probe, Laurdan, was also employed to assess changes in the lipid bilayer's structure following treatment. Laurdan, as described in Section 2.5.2, is well-established for its sensitivity to polarity relaxation within lipid environments, allowing for the evaluation of membrane hydration, water molecule movement, and the extent of ordering or disordering within the bilayer. Its fluorescence is influenced by the membrane's compactness or fluidity, providing insights into structural integrity [154,277,278]. By monitoring Laurdan's fluorescence, changes in the lipid bilayer's fluidity and order can be evaluated, complementing the findings from the BODIPY probe and offering a comprehensive understanding of the lipid membranes' response to oxidative treatment.

While BODIPY is suitable for monitoring oxidation rates during photocatalytic treatment, Laurdan cannot be used in the same way due to its susceptibility to degradation under oxidative conditions. Therefore, Laurdan was employed to assess structural changes in membranes, such as fluidity and hydration levels, at the end of the treatment with different photocatalytic agents, comparing results under light irradiation and with no exposure to light (dark). The samples were prepared in duplicate for each experimental set to distinguish the effect of photocatalytic activation.

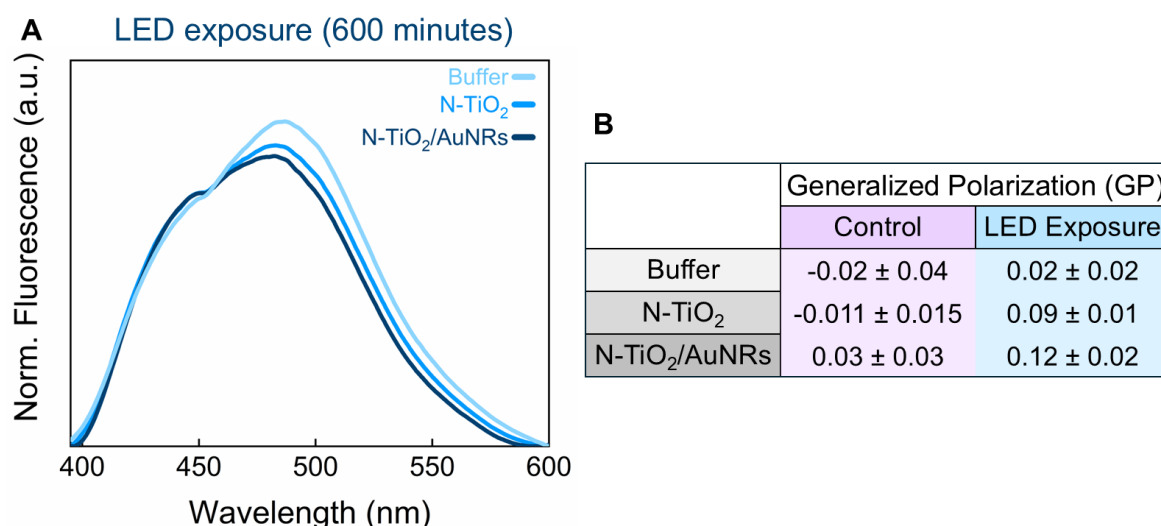


Figure 34: **Fluorescence emission spectra of Laurdan incorporated into lipid vesicles exposed to LED light for 600 minutes.** The spectra were obtained using an excitation wavelength of 380 nm ($\lambda_{exc} = 380$ nm) and fluorescence recorded in the range 400-600 nm. Spectra were corrected for instrumental response and normalized at 452 nm to better visualize the relative contributions of the two distinct phases of Laurdan's emission. The following conditions are shown: buffer (light blue), N-TiO₂ (blue), and N-TiO₂/AuNRs (dark blue). (A) Generalized polarization (GP) values for each condition under dark condition and upon LED exposure (B).

Figure 34A shows the spectra obtained for the samples exposed to LED light for 600 minutes. In comparison to the control condition, the fluorescence spectra of the different samples exhibit a noticeable transition to shorter wavelengths. For reference, spectra of the control condition (dark) are reported in Section 3.4. This spectral shift is particularly pronounced for the N-TiO₂ and N-TiO₂/AuNRs composites and is indicative of an increased order within the lipid bilayer. This

phenomenon aligns with the established effects of lipid oxidation, wherein unsaturated lipids undergo a transformation to their more rigid saturated counterparts. This oxidative transition reduces lipid mobility and consequently enhances the structural organization of the bilayer. This structural change is accompanied by the expulsion of water molecules from the lipid bilayer during the oxidation process, leading to dehydration [156,279]. The reduced water content decreases the dipolar relaxation, resulting in fluorescence emission at shorter wavelengths. These observations align with the mechanisms of lipid peroxidation and its impact on membrane dynamics. Similar results have been reported in studies such as that of Soto-Arriaza et al., where Laurdan fluorescence has proven effective in tracking progressive membrane rigidification at the interface between hydrophilic and hydrophobic regions following oxidation. The observed alterations mimic those caused by the incorporation of saturated lipids, which are known to decrease membrane fluidity and enhance bilayer rigidity [280].

Moreover, to quantify these differences, Figure 34B presents the corresponding generalized polarization (GP) values of Laurdan. For the control condition, the GP value is close to zero, indicating a relatively disordered membrane. Upon LED exposure, the GP values increase for all samples, with the greatest increase observed for the N-TiO₂/AuNRs composite, which shows a significant rise in GP from 0.03 ± 0.03 to 0.12 ± 0.02 . This increase in GP reflects the enhanced ordering of the lipid bilayer following photocatalytic activation, with the N-TiO₂/AuNRs composite exhibiting the most pronounced effect as compared to N-TiO₂ alone. This suggests that the incorporation of AuNRs enhances the photocatalytic activity, contributing to greater membrane rigidity and dehydration [149,152]. The most significant finding is that the photocatalytic treatment, regardless of the specific material, induces a general increase in membrane order, making the lipid bilayer more rigid and dehydrated.

These findings underscore the value of fluorescence spectroscopy as a tool for studying structural and dynamic changes in lipid membranes. This allows for gaining detailed insights into how photocatalytic treatment influences membrane organization, hydration, and fluidity, providing a non-invasive and quantitative approach to assessing membrane properties.

3.3 Conclusions

This chapter focused on the characterization of the spectroscopic and morphological aspects of nanostructures based on N-TiO₂, both individually and in combination with anisotropic AuNRs, forming the composite N-TiO₂/AuNRs. Both N-TiO₂ and AuNRs were synthesized following methodologies reported in the literature. The successful synthesis of these materials was confirmed through spectroscopic techniques. Morphological investigations were conducted using various electron microscopy techniques, including SEM, STEM, and TEM, to examine the structures across

different dimensional scales. N-TiO₂ displayed a heterogeneous morphology, forming micrometric aggregates composed of nanoparticle clusters, whereas AuNRs exhibited their characteristic rod-like anisotropic shape in the nanometric scale.

The composite material N-TiO₂/AuNRs, formed through the physical mixing of N-TiO₂ and AuNRs, demonstrated an effective method for combining these two materials. The surface interactions between the materials are facilitated by their opposing surface charges. The incorporation of AuNRs was strategically chosen to enhance the photocatalytic efficiency of N-TiO₂. The composite material displayed superior photocatalytic activity compared to N-TiO₂ alone, as evidenced by its performance in photodegradation studies of methyl orange, a model dye used in such experiments. The enhanced efficiency was attributed to the stabilization of photogenerated electron-hole pairs, likely resulting in a reduction in recombination events, which often limit the efficiency of photocatalytic processes.

In biological systems, DNA and lipid membranes were selected as critical targets for investigating the antibacterial potential of the photocatalytic materials. The N-TiO₂/AuNRs composite proved more effective in oxidizing DNA molecules. Spectroscopic analyses revealed an increase in oxidized nucleotide bases and a consequent unfolding of the DNA double helix. Additionally, as a proof of concept for biomaterial applications, a chitosan hydrogel incorporating the photocatalytic materials was fabricated. Even in this complex matrix, the materials demonstrated efficient DNA photodegradation, underscoring the robustness of the photocatalytic process.

Furthermore, fluorescence-based methods were employed to study lipid membrane oxidation using model vesicles labeled with two fluorescent probes, BODIPY and Laurdan. These probes provided critical insights into the rate and propagation of oxidation in vesicles treated with N-TiO₂ alone or the N-TiO₂/AuNRs composite. Once again, the composite exhibited superior efficiency and faster lipid oxidation rates. Additionally, it was possible to assess how membrane properties, such as rigidity and hydration levels, were affected by these treatments. Notably, treated membranes exhibited increased rigidity and dehydration, which are crucial findings for understanding membrane behaviour and advancing the development of novel antibacterial therapies.

3.4 Experimental Methodology

Materials

Titanium (IV) isopropoxide (TIPP, 97%), isopropanol ($\geq 99.8\%$), urea (cell culture grade), glacial acetic acid (AcOH, $\geq 99\%$), methyl orange (microscopy grade), hexadecyltrimethylammonium bromide (CTAB, $\geq 96\%$), calf thymus DNA (CT-DNA, fibrils, type V), chitosan, sodium acetate ($\geq 99\%$), sodium borohydride (NaBH₄), L-ascorbic acid (AA), silver nitrate (AgNO₃), and tetrachloroauric(III) acid (HAuCl₄ · 3H₂O). All were sourced from Sigma Aldrich. Lipids including 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC, 16:0–18:1), 1-palmitoyl-2-arachidonoyl-sn-phosphatidylcholine (PAPC, 16:0–22:4), and 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt) (POPG, 16:0–18:1), all with a purity exceeding 99%, were obtained from Avanti Polar Lipids (Alabaster, USA). Additional fluorescent dyes such as C11-BODIPY^{581/591} and Laurdan (6-dodecanoyl-2-dimethylaminonaphthalene) were procured from Thermo Fisher Scientific (USA). Ultrapure Milli-Q water (resistivity: 18.2 MΩ·cm, Smart2Pure filtration system, Thermo Fischer Scientific Inc.) was employed in all solution preparations.

The chemical syntheses for the preparation of N-TiO₂ and AuNRs have been extensively discussed in Chapter 2. The experimental procedure is briefly reported here.

Synthesis of N-TiO₂

Following a protocol from the literature [81,83], N-TiO₂ was synthesized via sol-gel method. Briefly, hydrolysis of the titanium precursor was initiated by adding 4 mL of TIPP dropwise to a 17.25 mL water-isopropanol mixture (10:1 v/v) under continuous stirring. Subsequently, a 10 M aqueous urea solution (3.75 mL) was gradually introduced to obtain a titanium-to-nitrogen (Ti/N) molar ratio of 1:6, forming a whitish slurry. The mixture underwent stirring for 4 hours, then was subjected to overnight drying at 100 °C. Subsequently, it was ground into a fine powder a calcination step in a muffle at the temperature of 400 °C was performed for 3 hours. Subsequently, the synthesized N-TiO₂ powder exhibits a characteristic yellow color. The purification from any residual reagents and impurities was performed by sequential sonication in acetone, ethanol, and Milli-Q water, each for 1 hour, and finally dried. For comparison, undoped TiO₂ was synthesized using the same protocol without urea. The materials' optical characteristics were examined through UV-Vis diffuse reflectance spectroscopy, (Jasco V-770 UV-Vis-NIR spectrophotometer with ISN-923 integrating sphere) in the 200–800 nm wavelength range.

Synthesis of AuNRs

Gold nanorods were synthesised through a seed-mediated growth approach as described in prior studies

[88,93,94,226]. The preparation of the seed solution involved combining 0.25 mM HAuCl₄ with 4.7 mL of 0.1 M CTAB, after which 300 µL of 0.01 M NaBH₄ was rapidly added as the reducing agent under continuous vigorous stirring. This process produced a brownish seed dispersion. Simultaneously, the growth solution was obtained by dissolving 0.025 mmol of HAuCl₄ in 50 mL of 0.1 M CTAB, to which 950 µL of 1 M HCl was subsequently added. Next, 600 µL of 0.01 M AgNO₃ and 400 µL of 0.1 M ascorbic acid were introduced in sequence, serving as the growth-directing agent and a mild reducing agent, respectively. As the final step, in the growth solution were added 120 µL of the freshly prepared seeds and incubated at 28 °C under controlled temperature conditions for 18 hours. The obtained violet CTAB-AuNRs colloidal suspension demonstrated long-term stability at room temperature for multiple months.

Preparation of N-TiO₂/AuNRs Composite

To prepare the composite material, CTAB-AuNRs were purified via three centrifugation cycles at 13,000 rpm for 10 minutes at 25 °C, using Milli-Q water to remove the CTAB stabilizing layer. The concentration of Au⁰ in the purified AuNRs suspension was quantified using UV-Vis absorption spectroscopy at 400 nm based on the formula [281]:

$$[Au^0] = \frac{Abs_{400\text{ nm}} \cdot 0.5}{1.2 \cdot 10^3}$$

A dispersion of N-TiO₂ was prepared in Milli-Q water and sonicated in the dark for 30 minutes. The AuNRs pellet was then resuspended in the N-TiO₂ dispersion at a ratio of 1:10 (mg/mL of N-TiO₂ to mM of Au⁰). The N-TiO₂/AuNRs composite was obtained by stirring the mixture in the absence of light for 30 minutes.

Dynamic Light Scattering (DLS) and Zeta Potential

The size and polydispersity of the AuNRs and lipid vesicles dispersed in various aqueous media were analyzed using the Malvern Zetasizer NanoZS. This device features a 632.8 nm laser and functions at a constant backscatter angle of 173°. The analyses were carried out at 25 °C after allowing the samples to equilibrate for 180 seconds. Each analysis represented the average of 10 individual runs, with each run lasting a specified duration. Zeta potential evaluations were performed at the same temperature using the same equipment.

Morphological Analysis via SEM, STEM, and TEM Techniques

The morphology of the materials was assessed through Scanning Electron Microscopy (SEM), Scanning Transmission Electron Microscopy (STEM), and Transmission Electron Microscopy (TEM). Additionally, their chemical composition was analyzed using Energy-Dispersive X-ray Spectroscopy

(EDX). SEM imaging was carried out with a Phenom ProX SEM microscope (Alfatest, Italy), utilizing an electron acceleration voltage of 15 kV. To prepare the samples, aqueous dispersions were deposited onto double-sided adhesive tape attached to a stainless-steel stub and dried before imaging. For the morphological analysis of AuNRs@CTAB, the CTAB surfactant was removed as detailed before, enabling clearer visualization of the nanoparticles.

STEM measurements were carried out using a STEM FEG Versa 3D–FEI microscope under an electron acceleration voltage of 30 kV. The samples were prepared by carefully applying the liquid dispersions onto 300-mesh copper grids coated with a thin carbon film. After deposition, the grids were left to air dry overnight to ensure the samples were ready for analysis.

Further morphological insights were obtained via TEM using a Philips CM 100 TEM microscope (Philips, The Netherlands) operating at 80 kV. Images were captured using the ITEM software suite. For this technique, 10 µl of each sample was subjected to negative staining and placed onto hydrophilic grids pretreated with glow discharge.

For STEM and TEM analyses, additional steps were taken to improve the dispersion of N-TiO₂ particles in solution, as their limited water solubility tends to promote aggregation. A stock dispersion of the particles was prepared in a 0.01 M HCl solution, followed by ultrasonication for 30 minutes at 5% amplitude to enhance dispersibility.

The size distribution of the N-TiO₂ particles and AuNRs were determined using ImageJ software by measuring the dimensions of at least 300 particles for each sample, analyzed from multiple images obtained from STEM data for N-TiO₂ and from TEM data for AuNRs. The data were then processed with the statistical tools in OriginLab to calculate and plot the particle size distributions, ensuring reliable and reproducible results.

UV-Vis-NIR Absorption Measurements

UV-Vis-NIR absorption spectra were obtained utilizing a Jasco V-770 spectrophotometer. All measurements were performed at ambient temperature using quartz cuvettes with a 1 cm optical path length. Data were collected across the 200–900 nm wavelength range, with a scanning rate of 100 nm/min, a response time of 1 second, and a spectral bandwidth of 2.0 nm.

Photocatalysis Experiments

To evaluate the photocatalytic performance of N-TiO₂ nanoparticles, experiments were designed to investigate their efficiency in degrading organic pollutants, specifically targeting Methyl Orange (MO), a common dye in wastewater. Photocatalysis, a process that accelerates chemical reactions through light absorption, is widely recognized for its potential in environmental remediation. Following a protocol previously reported by Ferrara et al. [83] the photocatalytic performance of N-

TiO₂ was assessed under illumination from a blue LED light source, centered at 420 nm. This light source was provided by EmoLED (Lumileds, L1CU-VLT1000000000), operating within the 400–450 nm spectral range. The LED power at a 2 cm distance was calibrated to 8 mW/cm² using a silicon cell and tester. The measurements were conducted with and without AuNRs to evaluate possible synergistic effects on the photocatalytic process.

N-TiO₂ (10 mg/mL) and N-TiO₂/AuNRs were dispersed in an aqueous solution of MO (50 mg/L), a standard model compound for photocatalytic degradation studies. To ensure consistency, all photocatalytic materials were used at concentrations providing equivalent amounts of N-TiO₂. Before illumination, samples were stirred for 30 minutes in dark conditions at room temperature to allow MO adsorption on the photocatalyst surface, and an initial absorbance spectrum ($t = 0$) was recorded. Illumination exposures were performed through a sealed Pyrex beaker, ensuring continuous magnetic stirring to maintain homogeneous exposure and facilitate uniform reaction conditions as shown in Figure 35. The LED source was activated at least 30 minutes before starting illumination to stabilize its output.

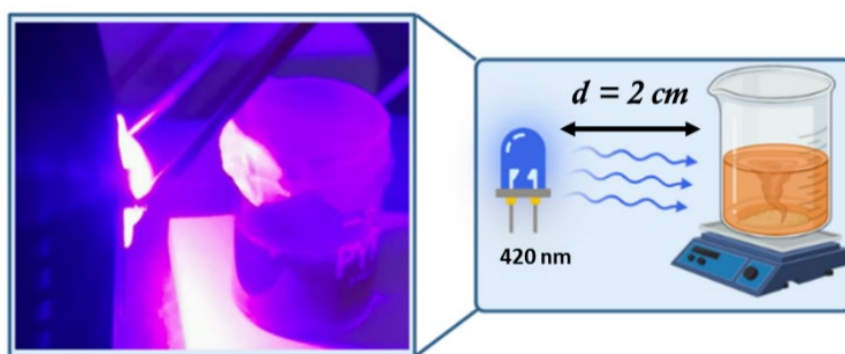


Figure 35: Illustration of the experimental setup for photodegradation studies. The setup includes a beaker containing the methyl orange solution placed under a blue LED light source emitting at 420 nm, with a light-to-sample distance of 2 cm. The solution is stirred continuously during irradiation to ensure uniform exposure. At specific time intervals, aliquots are taken from the solution, and the absorbance is measured. Created with Biorender.com

Irradiation intervals of 0.5, 1, 1.5, 2, 3, and 4 hours were employed to monitor MO degradation over time. At each interval, aliquots of the sample were centrifuged to separate the photocatalyst pellet, and the supernatant was analyzed using UV-Vis spectrophotometry to determine MO concentration based on absorbance. After measurement, the supernatant was returned to the reaction vessel to continue the experiment. Control experiments were performed under identical conditions without illumination, as well as in the absence of N-TiO₂ under visible light, confirming that the observed photocatalytic activity was attributable to N-TiO₂.

Additionally, the same experimental protocol was applied to examine the photodegradation of calf thymus DNA (CT-DNA) in MilliQ water at a concentration of 0.1 mM. Freshly prepared CT-DNA

solutions were characterized by measuring absorbance at 260 nm using a molar extinction coefficient of 6600 L mol⁻¹ cm⁻¹ [64,282].

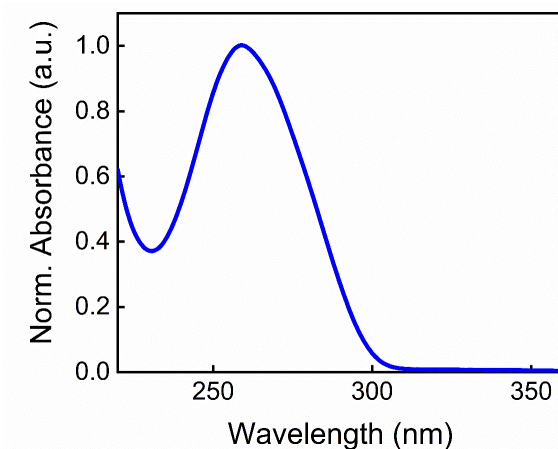


Figure 36 - *UV-Vis absorption spectrum of CT-DNA in aqueous solution, recorded in the range of 220–360 nm, normalized at the maximum absorption peak.*

The characteristic absorption spectrum of CT-DNA is shown in Figure 36 highlighting the prominent absorption peak at 260 nm, which is a characteristic feature of DNA. This peak corresponds to the absorption of UV light by the aromatic bases in DNA and is widely utilized to estimate DNA concentration and purity. The observed cutoff in absorbance beyond 300 nm is consistent with the intrinsic absorption properties of DNA, as its nucleobases do not absorb light at longer wavelengths. The intrinsic photostability of a CT-DNA solution under blue light irradiation was also investigated to exclude effects light-related.

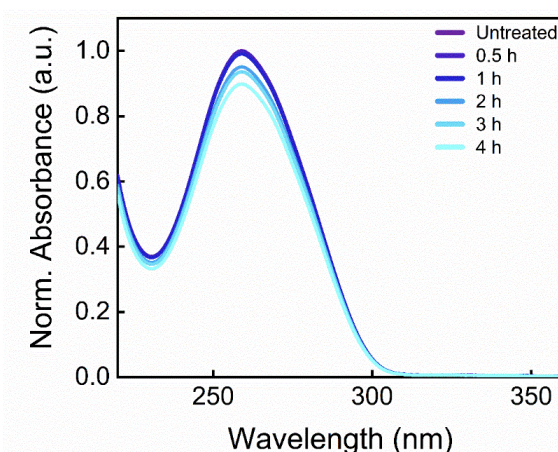


Figure 37: *Normalized UV-Vis absorption spectra of CT-DNA solution exposed to blue light irradiation for up to 4 hours. Spectra are normalized to the maximum of the untreated sample.*

The spectra in Figure 37 reveal that the characteristic absorption peak at 260 nm remains largely unchanged, with a slight reduction observed. This negligible decrease confirms the overall photostability of the CT-DNA solution, ensuring that the more pronounced effects in subsequent experiments involving N-TiO₂ treatment can be attributed to the photocatalytic process rather than

light exposure alone.

Each experiment, including those involving MO and CT-DNA, was conducted in triplicate to ensure reproducibility and reliability. In Figure 38 is reported an illustration to summarize our experimental approach for evaluating photodegradation effects on MO and CT-DNA in solution.

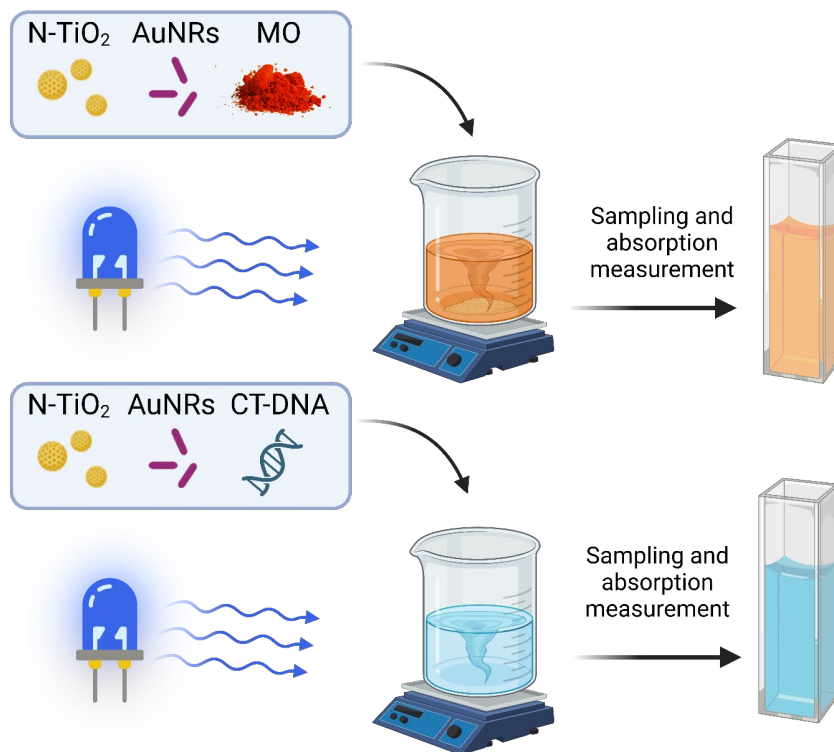


Figure 38: **Experimental schematic for photocatalytic activity studies.** The top panel illustrates the setup for MO photodegradation, where the photocatalyst ($N\text{-TiO}_2/\text{AuNRs}$) is dispersed in an MO solution and exposed to blue LED light. The solution is stirred during irradiation, and aliquots are collected for absorbance measurements. The bottom panel represents the setup for CT-DNA photodegradation, with a similar process involving $N\text{-TiO}_2/\text{AuNRs}$ in a CT-DNA solution. Both setups utilize UV-Vis spectrophotometry to monitor changes in optical properties, reflecting the degradation efficiency of the respective substrates. Created with Biorender.com

Preparation of Chitosan Hydrogels

Chitosan-based hydrogels were prepared as a class of hydrophilic biomaterials distinguished by their exceptional ability to absorb water and remain in a swollen state without dissolving. The preparation of these hydrogels was specifically designed to incorporate different materials, aiming to enhance their properties for targeted biomedical applications.

To prepare chitosan hydrogels, a 3% w/v chitosan solution was first dispersed in 1% v/v acetic acid. Cross-linking was achieved by mixing the chitosan solution with EPEG in a 1:1 volumetric ratio, followed by heating at 65°C for one hour, which facilitated the formation of a stable hydrogel network, as reported by Ferrara et al. [83]. For the incorporation of $N\text{-TiO}_2$ into the chitosan matrix, the photocatalytic powder was added to the chitosan solution before the addition of EPEG. The mixture was vortexed thoroughly to ensure uniform dispersion of $N\text{-TiO}_2$ within the hydrogel. The as-prepared hydrogel was subsequently soaked overnight in an aqueous solution containing the target molecule,

CT-DNA (2 mg/ml), or water as a reference. This step enabled effective swelling and loading of the target molecules. A reference hydrogel, without N-TiO₂, was prepared following the same protocol. Additionally, chitosan hydrogels were prepared incorporating the composite N-TiO₂/AuNRs. Prior to incorporation, the AuNRs underwent a purification process involving two centrifugation steps at 13,000 rpm for eight minutes at 25°C to remove residual CTAB. The resulting pellet was resuspended, and the absorption spectrum was analyzed to determine the concentration of Au⁰ at 400 nm. Following dilution, a final centrifugation step yielded a concentrated AuNRs pellet, which was then combined with N-TiO₂, left to stir for 30 minutes and then mixed in the 3% (w/v) chitosan solution prior to the addition of EPEG. The resulting hydrogel, characterized by a pinkish color, confirmed the successful incorporation of AuNRs.

Preparation of Small Unilamellar Vesicles (SUVs)

The preparation of Small Unilamellar Vesicles (SUVs) was carried out using the dry lipid film (DLF) technique as elsewhere described [269,270]. Briefly, the procedure started from the stock solutions of POPC and PAPC at a concentration of 10 mg/mL, which were prepared in chloroform, whereas the POPG stock solution was prepared in a solvent mixture of chloroform and methanol in a 3:1 volume ratio. These stock solutions were stored in an inert nitrogen atmosphere, shielded from light exposure, and kept frozen until required.

To create the desired lipid mixture with a molar ratio of POPC:PAPC:POPG at 50:25:25, appropriate volumes were withdrawn from each stock solution. The solvent was evaporated under a nitrogen stream, followed by an additional drying period of two hours under vacuum to ensure complete elimination of chloroform. This process resulted in the formation of a thin lipid film along the inner surface of the vial.

The dry lipid film was rehydrated with a 10 mM acetate buffer at pH 5.4. Vortexing and sonication were employed to ensure thorough hydration and detachment of the lipid film from the vial wall, resulting in the formation of Multilamellar Vesicles (MLVs). These MLVs were further processed by extrusion through a 100 nm polycarbonate membrane using an Avanti Mini-Extruder (Avanti Polar Lipids, Inc). This procedure was repeated for 31 cycles to yield SUVs with uniform size distribution [269,272]. The freshly prepared SUVs were stored under nitrogen, in a dark and refrigerated environment throughout the experimental period, to preserve their stability. Ensuring their stability was critical, with fresh preparations made for each experiment. The size distribution of the vesicles was analyzed using Dynamic Light Scattering (DLS).

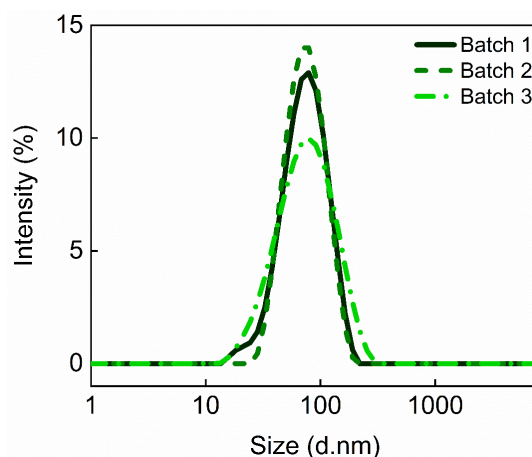


Figure 39: **Size distribution profiles of SUVs measured by DLS.** The graph shows the Z-average size for three independent batches.

The data illustrate the reproducibility of the production process for SUVs (Figure 39). The size distribution profiles for three independent batches are overlaid, demonstrating a highly consistent Z-average size of approximately 100 nm. The tight distribution and the absence of significant variation between batches confirm the robustness and reliability of the preparation method.

The Z-average size of ~100 nm falls within the optimal range for SUV models, making these vesicles particularly suitable for studies of oxidative degradation. This size is advantageous because it represents a biologically relevant dimension for mimicking lipid-based vesicular structures, facilitating the investigation of oxidative processes under controlled experimental conditions [283,284]. Additionally, the uniformity of the vesicle size is critical to ensure consistency in experimental outcomes, particularly in our studies, where particle size and surface area influence the rate and extent of oxidative degradation reactions. For optimal performance, the SUVs were utilized within two days of preparation.

Lipid Peroxidation Fluorescence Assay

To assess the rate of oxidation in lipid membranes, SUVs suspensions with a lipid concentration of 5 mg/mL (650 μ M) were labeled with the fluorescent dye C-11 BODIPY^{581/591} (molar extinction coefficient of 139,444 $\text{M}^{-1} \text{cm}^{-1}$ at 581 nm [140]). Specifically, BODIPY probe from a stock solution of 200 μ M in DMSO was added to the SUV suspension (70 μ L) to achieve lipid-to-dye molar ratio of 0.03%.

To ensure the effective incorporation of the dye into the lipid bilayer, the samples were equilibrated for two hours prior to experimentation. During this period, the fluorescence emission of the dye stabilized, establishing the baseline for subsequent measurements. It was assumed that the dye was fully integrated into the bilayer and that free dye contributed minimally to the signal due to its hydrophobic properties, which significantly reduce its emission in aqueous environments.

Subsequently, the labeled SUVs were combined with dispersions of 100 ppm dispersions of N-TiO₂, N-TiO₂/AuNRs, and acetate buffer as a control, in a final volume of 3 mL. The N-TiO₂/AuNRs composite was prepared according to a previously established protocol, ensuring that the N-TiO₂ concentration was equivalent to 10 mg/mL in the final dispersion.

Fluorescence measurements were conducted with a Jasco FP-8500 spectrofluorometer, integrated with a Jasco ETC-815 Peltier temperature controller set to 25°C. For the BODIPY assay, the excitation wavelength was set to 510 nm, while emission spectra were acquired within the 515–650 nm range, with excitation and emission bandwidths of 5 nm, with a response time of 1 second, a data acquisition interval of 0.5 nm, and a scanning speed of 100 nm/min. Measurements were performed, under stirring (150 rpm) every 5 minutes for up to 10 hours, with a continuous illumination of the sample by a blue LED placed above the cuvette (1 cm path, quartz) within the spectrofluorometer chamber.

Lipid Membrane Order Fluorescence Assay

The structural order of lipid membranes was investigated using the fluorescent probe Laurdan. SUVs suspension (70 µL from stock suspension 650 µM) was incubated with 100 ppm dispersions of N-TiO₂, N-TiO₂/AuNRs, AuNRs alone, and buffer as a control in a final volume of 3 mL. Each sample set was prepared in duplicate: one set was exposed to continuous illumination by a blue LED under gentle stirring overnight, while the other set was stirred in darkness to prevent light exposure.

Following the treatments, Laurdan fluorescence emission was measured to evaluate changes in the lipid order and disorder. Laurdan was prepared as a 0.5 mM stock solution in DMSO (molar extinction coefficient of 19,500 M⁻¹ cm⁻¹ at 366 nm [277,285]). Laurdan from the stock solution was added to the SUV suspensions after treatment, to achieve a lipid-to-dye molar ratio of 0.07%, and samples were allowed to equilibrate for three hours before measurements to achieve stable incorporation of the dye. Fluorescence spectra were collected using the same spectrofluorometer and parameters as described above, except that the excitation wavelength was set to 380 nm and spectra were acquired over the 385–600 nm range. The emission spectrum of Laurdan varies with the membrane phase state, showing an emission peak at 440 nm when in the gel phase and shifting to 490 nm in the liquid-crystalline phase.

The control sample was kept in the dark for 600 minutes under stirring, either in contact with the photocatalytic material or in buffer alone. After the 600-minute exposure, the Laurdan probe was added to the vesicles, providing insights into the order/disorder state of the lipid bilayer. The observed changes in the vesicles were attributed solely to interactions with the photocatalytic material or to the natural evolution of the vesicles during this period, and not to external factors.

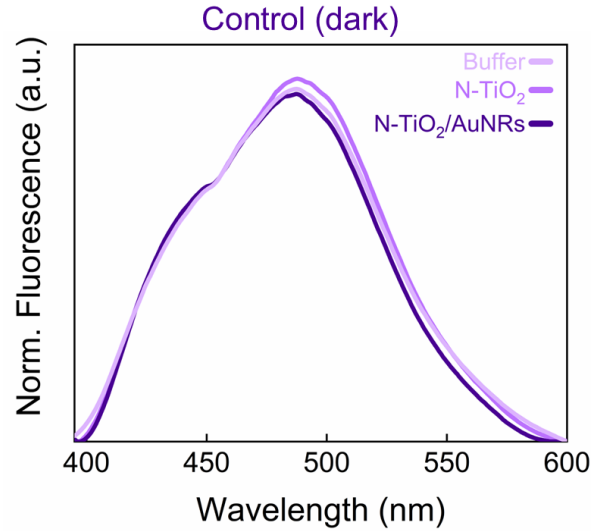


Figure 40: **Fluorescence emission spectra of Laurdan incorporated into lipid vesicles under dark conditions (control).** The spectra were recorded with excitation at $\lambda_{exc} = 380$ nm and fluorescence recorded in the range 400-600 nm. Spectra were corrected for instrumental response and normalized at 452 nm to better visualize the relative contributions of the two distinct phases of Laurdan's emission. The following conditions are shown: buffer (light purple), N-TiO₂ (purple), and N-TiO₂/AuNRs (dark purple) after a 600-minute incubation.

Figure 37 presents the fluorescence emission spectra of Laurdan incorporated into the lipid vesicles without any exposure to light (dark conditions). A prominent peak centered around 490 nm is observed for all samples, indicating a disordered membrane state. This fluorescence emission is affected by the hydration level of the lipid bilayer. As membrane hydration and disorder increase, Laurdan's fluorescence exhibits a red shift, which is attributed to dipolar relaxation occurring in its excited state [148,286,287].

The spectra for the N-TiO₂ and N-TiO₂/AuNRs samples are similar to the buffer control, with no significant shift in the emission maximum or intensity. This suggests that, under dark conditions, the vesicles remain in a similar disordered state, and no substantial structural changes occur in the lipid bilayer due to the presence of the photocatalytic material alone. Therefore, these data serve as a baseline for understanding the membrane structure before the photocatalytic treatment, ensuring that any observed changes in the order/disorder of the membrane can be attributed to the photocatalytic activation and not to inherent properties of the materials used.

This phase-dependent fluorescence behaviour allowed for the assessment of membrane dynamics using the Generalized Polarization (GP) index, calculated with the formula proposed by Parasassi and Gratton [149,154,279]:

$$GP = \frac{I_{440} - I_{490}}{I_{440} + I_{490}}$$

Where I_{440} and I_{490} represent the fluorescence intensities measured at 440 nm and 490 nm, respectively.

Chapter 4

Fabrication and Characterization of Waterborne Electrospun Lignin Nanofibers as Advanced Drug Delivery Systems

This chapter describes the production of lignin-based nanofibers using a minimalist and eco-friendly approach through waterborne electrospinning, avoiding the use of toxic organic solvents. Nanofibers were fabricated at different lignin concentrations, and their morphology and structural features were thoroughly characterized using high-resolution techniques. The intrinsic autofluorescence of lignin was observed through two-photon excitation to gain deeper insights into their 3D organisation in a non-invasive method that does not require staining or other preparation treatments. Particular attention was given to understanding the relationship between molecular structure and physicochemical properties, focusing on how lignin concentration in nanofibers affects their morphology, mechanical properties and antioxidant activity. Finally, the potential applications of these nanofibers were investigated, including their ability to dissolve in water and serve as tuneable drug delivery system (DDS) for antibacterial agents.

4.1 Introduction

4.1.1 Composition, Challenges, and Valorization of Lignin

As the second most prevalent biopolymer after cellulose, lignin represents a highly available natural resource and a major component of wood. However, due to its complex molecular organization and challenging processability, it is often disposed of as industrial waste. While 98% of the over 50 million tons produced annually is used for combustion and power generation, only 2% is applied in low-value uses such as fillers, coreactants and adhesives [288,289]. This practice underscores the need for innovative strategies to enhance its valorization.

Lignin holds significant potential for biomedical applications owing to its abundance, low cost, biodegradability, and intrinsic properties including UV-blocking, antioxidant activity, antibacterial effects, and biocompatibility. [290,291]. Additionally, lignin has shown potential to be used also as a photocatalyst due to its aromatic structure, by absorbing light and facilitating electron transfer. To this end, Lignin-based materials have been effectively used as photocatalysts under light irradiation,

demonstrating high efficiency in dye degradation [292,293]. These characteristics make lignin a promising candidate for diverse applications, including, drug delivery platforms, wound healing dressings, and biosensors. However, its utilization faces significant challenges due to its high heterogeneity, resulting from variations in its botanical sources and the extraction techniques employed [294].

At the molecular level, lignin is composed primarily of three aromatic monomers: *p*-coumaryl, coniferyl, and sinapyl alcohols (Figure 41). The relative quantities and proportions of these units vary depending on the source. The high content of aromatic groups, while advantageous for certain applications, also contributes to its limited water solubility and the propensity to form aggregates due to strong π - π interactions [295,296].

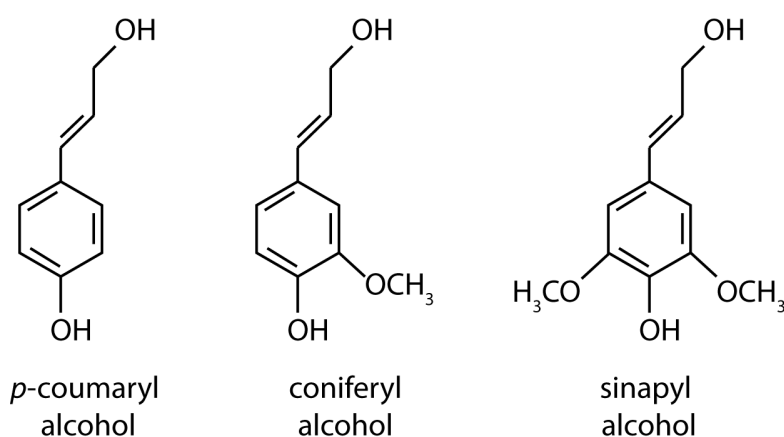


Figure 41: Chemical structures of the three primary lignin monomers: *p*-coumaryl alcohol (H), coniferyl alcohol (G), and sinapyl alcohol (S) [297,298]. These monomers vary in methoxylation, with *p*-coumaryl alcohol lacking methoxy groups, coniferyl alcohol having one, and sinapyl alcohol having two, contributing to lignin's structural diversity.

Lignin is first separated from cellulose through physical or chemical extraction methods, which can either involve sulfur or be sulfur-free. Based on these methods, lignins are categorized into four distinct types: Kraft lignin, lignosulfonates, soda lignin, and organosolv lignin. Among these, Kraft lignin is the most abundant and cost-effective type, extracted through the efficient Kraft process. It has higher molecular weight and is less chemically modified compared to other types making it suitable for various applications [299]. These physicochemical characteristics restrict its direct application and necessitate chemical or enzymatic modifications to improve its functionality.

To address these challenges, functionalization strategies are commonly employed, but also chemical fragmentation techniques and enzymatic treatments, such as laccase-based processes, which can break lignin into smaller, more manageable units, improving its solubility and reactivity. In particular, functionalization is a very usable strategy due to the presence of various functional groups, including methoxys, phenolic hydroxyls, and carbonyl groups [300].

Another promising approach involves the production of lignin-based nanoparticles or nanofibers aiming to enhance lignin's versatility but also enabling its use as a carrier for therapeutic agents, creating synergistic effects that combine lignin's intrinsic properties with those of the delivered molecules [301,302].

While the use of organic solvents and complex treatments can significantly improve lignin's processability, these strategies often compromise environmental sustainability. In particular, solvents with intermediate polarity or binary systems are more suitable [303]; however, these systems are frequently volatile, flammable, explosive, and toxic [304]. Thus, increasing attention is being directed toward the development of environmentally friendly and sustainable approaches for lignin modification and utilization, such as employing renewable solvents, bio-based catalysts, and energy-efficient processes. The development of scalable, environmentally friendly methodologies for lignin valorization would not only unlock its potential as a valuable resource but also contribute to reducing reliance on fossil-based materials and advancing circular bioeconomy goals. Further research into the optimization of extraction techniques and functionalization methods is essential to fully exploit this underutilized biopolymer.

4.1.2 Lignin-based Nanofibers

Electrospun nanofibers are among the most promising nanostructures due to their potential to form three-dimensional scaffolds with a nanometric structure. These scaffolds offer high tunability, making them suitable for several applications, including tissue engineering, drug delivery, and wound treatment. In this context, the unique characteristics of lignin, including its aromatic structure and high mechanical strength, make it an attractive material for nanofiber production [305,306].

However, pure lignin has limitations in electrospinning, particularly due to its poor solubility and low electrospinnability [307]. To address these limitations, lignin is commonly blended with other polymers. The most commonly used ones include polyvinyl alcohol (PVA), polyvinyl pyrrolidone (PVP), and poly(lactic acid) (PLA). An example of this approach is the development of lignin-PMMA copolymers, which were blended with poly(ϵ -caprolactone) (PCL), dissolved in a chloroform/methanol mixture and subsequently electrospun into nanofibers. The incorporation of these copolymers significantly improved the mechanical behaviour and the biocompatibility of the fibers [308]. In another case, PLA-lignin copolymers, mixed with poly(L-lactide) (PLLA), were dissolved in HFIP and electrospun into nanofibers with diameters of 350–500 nm. These fibers exhibited strong antioxidant properties, scavenging radicals for over 72 hours. Moreover, the lignin-containing fibers enhanced cell proliferation, particularly protecting cells from oxidative stress [309]. Despite the promising outcomes of these studies, these blends rely on the use of organic solvents, such as

dimethylformamide (DMF), acetone, methanol, chloroform, or hexafluoroisopropanol (HFIP). But their environmental impact remains a concern, as they are often toxic, flammable, and challenging to dispose of, contributing to environmental pollution and health risks [310–312]. Therefore, ongoing research focuses on identifying greener solvents and sustainable approaches to reduce these negative effects while maintaining the improved processability and mechanical properties achieved through polymer blending [104,108,313].

To the best of our knowledge, the electrospinning of lignin blended with PEO in aqueous alkaline solutions has been explored in only one study, reported by Poursorkhabi et al. [314]. Their work focused on using softwood Kraft lignin (Indulin AT) and high-molecular-weight PEO under highly alkaline conditions ($\text{pH} > 13$), primarily for the production of fibers intended for carbonization. While their approach demonstrates the feasibility of aqueous electrospinning, the use of highly alkaline conditions ($\text{pH} > 12$) may profoundly influence different cell types, such as keratinocytes and fibroblasts, affecting their viability and proliferation capacity [315], thereby limiting its applicability in biomedical contexts. Moreover, their production of nanofibers was not aimed at biomedical purposes, so this aspect was not studied. Notably, the softwood lignin they used, such as Indulin AT, is known to contain a higher amount of guaiacyl units, resulting in a more condensed and hydrophobic structure, which further reduces performance compared to alkali Kraft lignin, which has already been partially treated [316].

In the present thesis, green and sustainable strategies are explored to tailor the process for biomedical applications, addressing these limitations. Specifically, alkali Kraft lignin is utilized, employing sustainable dispersion techniques such as ultrasonication and optimizing pH conditions to 11, thereby reducing the harshness of the alkaline environment while maintaining process efficiency and material compatibility for biomedical contexts.

4.2 Experimental Results and Discussion

4.2.1 Improving Water Solubility of Lignin through Sustainable Strategies

In the quest for sustainable solutions to improve the solubility of lignin, several approaches have been explored. Traditional methods often rely on the use of organic solvents or complex chemical treatments to enhance lignin's dispersibility and solubility [317–319]. These techniques, although effective, are often criticized for their environmental impact, primarily due to the toxicity of the solvents involved and tend to involve a series of steps that require careful control of reaction parameters, including factors like temperature and the choice of solvent [288,295,304]. The complexity of these methods, combined with the need for post-treatment steps such as solvent recovery and disposal, makes them

more challenging to implement on a large scale. In contrast, more minimalist approaches that aim to exploit lignin's intrinsic properties can offer a simpler, more straightforward solution. One such approach involves modulating the pH of the solution to alkaline levels, typically above pH 10, employing sodium hydroxide or similar agents. Indeed, it is well known that the solubility of lignin improves significantly in alkaline conditions due to the deprotonation of phenolic hydroxyl groups (-OH) within its structure [320]. This change increases lignin's affinity for water molecules, offering a straightforward and effective method for enhancing its solubility without the need for harsh chemical treatments.

This comprehension provides the foundation for exploring simpler, more sustainable strategies to enhance lignin's solubility. For this purpose, the lignin's spectroscopic properties were evaluated. The UV-Vis spectrum, shown in Figure 42, reveals key absorption features that are characteristic of chromophoric groups and conjugated structures within lignin.

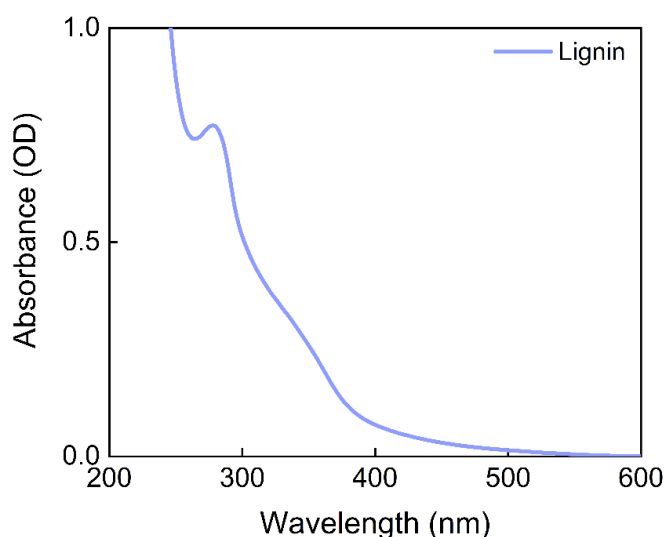


Figure 42: *UV-Vis absorption spectrum of an aqueous Kraft Lignin dispersion measured in the range of 200–600 nm. The spectrum was obtained from a sample prepared by diluting a 10% lignin dispersion, resulting in a final concentration of lignin of 0.1 mg/mL.*

The primary shoulder at 280 nm is associated with π - π^* electronic transitions of aromatic rings, linked to non-conjugated phenolic groups. These groups, found in guaiacyl (G) and syringyl (S) units, suggest a G/S-rich lignin profile. A secondary shoulder, observed between 316–320 nm, corresponds to n - π^* transitions associated with conjugated phenolic structures [321]. Notably, beyond 350 nm, is possible to distinguish the increase in turbidity, likely due to light scattering caused by suspended particles or aggregates in the solution. This effect highlights the potential presence of insoluble lignin fragments, as expected.

The solubility of lignin in relation to pH was also examined by measuring the concentration of

dissolved lignin, determined through absorbance at 280 nm. The analysis started with 10% w/w lignin dispersion, which had an initial pH of 9.7, as indicated by the green circle in Figure 43.

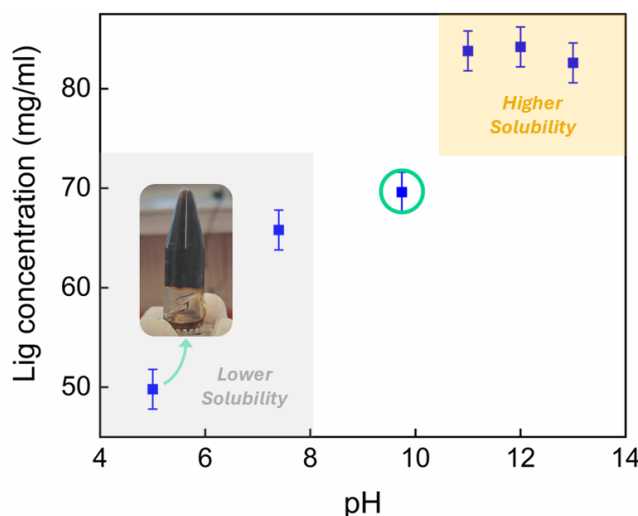


Figure 43: **Solubility of lignin as a function of pH.** The plot illustrates the relationship between lignin solubility (mg/mL) and pH, as measured via absorbance at 280 nm. The starting pH of the dispersions was approximately 10, highlighted by the green circle. Adjustments to the pH show a significant impact on lignin solubility. Two boxes are included to indicate the regions where solubility is higher (yellow box) and lower (gray box), respectively. At pH 5, lignin precipitates and forms a gel-like structure, as demonstrated by the inverted tube shown in the inset image.

From this starting point, the pH was titrated both upward and downward to monitor the changes in lignin solubility in real time. The results demonstrate that solubility increases significantly with rising pH, reaching its maximum at pH 11. This condition was subsequently chosen for further processing steps due to the high solubility observed. Additionally, as the pH decreased to 7 and 5, the solubility of lignin dropped drastically, resulting in precipitation and the development of a dense gel-like structure, as shown by the inverted tube in the inset image.

The changes in lignin solubility are highly related to its isoelectric point (pI), which, as a polyelectrolyte, is influenced by acidic functional groups like carboxyl (-COOH) and sulfonate (-SO₃H) groups. At high pH values, these groups dissociate, forming negatively charged species and enhancing solubility due to increased electrostatic repulsion. However, as the pH approaches acidic conditions, these groups progressively lose their charge, reducing electrostatic repulsion and promoting aggregation and gelation [322]. The isoelectric point of lignin is typically observed at low pH values, though its precise location depends on the composition and type of lignin. Several studies place the isoelectric point in the acidic range, with values reported between 1 and 5, reflecting differences in the proportion and type of functional groups present in different lignin samples [323–325]. Conversely, increasing the pH to 11, 12, and 13 enhances solubility, which can be ascribed to the deprotonation of -OH and -SO₃H functional groups. Based on the results, we selected pH 11 for subsequent experiments, as it provided the optimal solubility required for producing stable and

homogeneous dispersions suitable for electrospinning.

To further enhance the processability of the lignin dispersions, we combined the pH adjustment approach with ultrasonication to achieve more uniform and stable dispersions. Ultrasonication, represents indeed an environment-friendly approach, which avoid the need for hazardous chemicals and reduces particle size by breaking cross-links in polymeric materials through intense mechanical shear forces [326]. Unlike previous studies that relied on highly alkaline pre-adjusted solutions to maximize lignin solubility, often aimed at producing lignin-based fibers for other applications including systems for energy conversion and storage [314,327,328], the here presented work utilized a dynamic titration approach. This method allowed the identification of the minimally alkaline pH required for subsequent processes. This optimization is particularly significant for biomedical applications, which require careful consideration of conditions that may differ from those in non-biomedical contexts. Specifically, in the design and development of this biomaterial, it was taken into account that, in the case of using these nanofibers in DDS, there are additional constraints regarding the pH range to be employed. For instance, a pH value above 11 in the suspension could compromise the chemical stability of certain drugs. Consequently, targeted approaches must be implemented to ensure that the system is suitable for the stable and effective administration of drugs..

4.2.2 Production of Lignin Nanofibers by Waterborne Electrospinning

Waterborne electrospinning relies on water as the only solvent, which as discussed in Section 2.2.1, may pose significant challenges to process stability. This is particularly evident when compared to conventional methods that rely on the use of organic solvents whose properties facilitate fiber formation. The characteristics features of water, including its high surface tension and low volatility, contribute to a less stable spinning process, necessitating careful optimization of key parameters [104,105]. Hence, a waterborne electrospinning protocol was developed and optimized to address these challenges, employing a minimalist approach that combines titration to a alkaline pH and ultrasonication. The results of these optimizations, demonstrating the impact of pretreatment on the stability and reproducibility of the process, are presented in Figure 44 where representative SEM images are shown to evaluate the impact on the morphology of lignin nanofibers. Specifically, the optimized procedure involved preparing a lignin dispersion at a 10% weight concentration and adjusting its pH to 11 before subjecting it to ultrasonication for 30 minutes to enhance dispersibility. After this pretreatment, the lignin dispersion was mixed with a 4% weight PEO dispersion in a 1:2 ratio, resulting in a final lignin content in the fibers of 56% w/w.

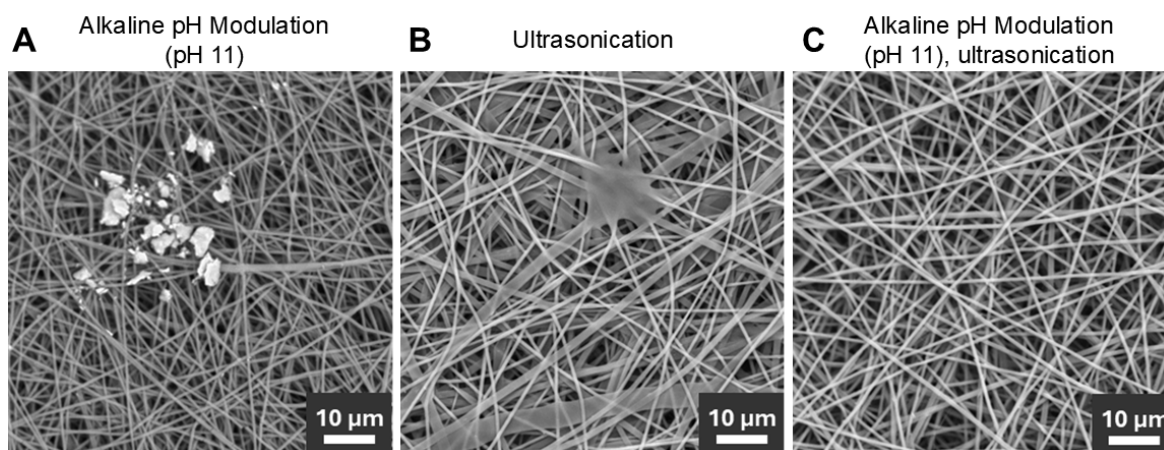


Figure 44: Representative SEM images of lignin nanofibers produced under different lignin dispersion pretreatment conditions prior to mixing with PEO and electrospinning. All samples were prepared from a 10% lignin aqueous dispersion mixed with a 4% PEO solution in a 1:2 ratio, resulting in a final lignin content of 56% w/w in the fibers. (A) fiber produced from lignin aqueous dispersion at pH adjusted to 11 from its initial value of 9.7, prior to spinning, showing the presence of aggregates. (B) Fibers produced from a lignin dispersion subjected only to ultrasonication at its initial pH of 9.7, resulting in fused and non-uniform fibers. (C) Fibers produced from a lignin dispersion initially at pH 9.7, adjusted to pH 11 and then ultrasonicated, yielding homogeneous and defect-free fibers.

In Figure 44A, the nanofibers produced from a lignin dispersion initially at pH 9, adjusted to pH 11 prior to electrospinning, are not completely uniform, showing aggregates, indicating incomplete dispersion stability.

In line with previous studies on the use of ultrasonication to enhance the electrospinning of biological molecules such as plant proteins (e.g., soy, pea) and other proteins like α -lactalbumin [105,329,330], we investigated its potential to improve lignin dispersion alongside pH adjustment for the waterborne electrospinning process. As shown in Figure 44B, the ultrasonicated lignin dispersions did not show aggregates. However, the electrospinning process still exhibited significant irregularities, with frequent clogging that obstructed the needle, leading to instability. The fibers tended to fuse together, and occasional fusion points were observed. These points were likely caused by irregular jets. Indeed, dispersion of droplets, not fully evaporated, may reach the collector while still wet, eventually melting the fibers in the process. This resulted in an unstable Taylor cone, which hindered the ability to electrospun uniformly. The best results were obtained from ultrasonication of dispersions at pH 11 (Figure 44C). This sample displayed the most homogeneous and defect-free fibers, demonstrating the synergistic effects of these pre-treatment methods in maximizing lignin dispersibility and stabilizing the electrospinning process.

Following the same process analogous protocol, varying experimental conditions including the applied voltage (kV), solution flow rate (ml/h), and needle-to-collector distance (cm), were adjusted to fabricate lignin-based electrospun fibers aimed at maximizing lignin content. Table 1 summarizes a selection of successfully produced samples, highlighting the lignin and PEO content (% w/w) in the

nanofibers as well as the corresponding electrospinning parameters used. This overview demonstrates the interplay between composition and process parameters in achieving stable and reproducible fiber production.

Sample name	Content in nanofiber % (w/w)		Electrospinning Parameters		
	Lig	PEO	Voltage applied (kV)	Flow rate (ml/h)	Needle-to-collector distance (cm)
<i>Lig-56%</i>	56	44	17.4	0.4	20
<i>Lig-48%</i>	48	52	17.4	0.9	15
<i>Lig-38%</i>	38	62	17.4	0.9	15
<i>Lig-24%</i>	24	76	17.5	0.9	16
<i>PEO</i>	0	100	18	0.7	15

Table 1: Composition and Electrospinning Parameters of Lignin/PEO Nanofiber Samples. Weight percentage (w/w) of lignin (Lig) and polyethylene oxide (PEO) in the nanofiber samples, and electrospinning parameters, such as applied voltage, flow rate, and needle-to-collector distance used for each sample preparation.

Multiple experiment sets have shown that samples with higher lignin content, such as Lig-56%, required a lower flow rate (0.4 ml/h) and a larger needle-to-collector distance (20 cm) to prevent clogging and ensure stable fiber deposition. At lower lignin content, probably due to differences in viscosity and conductivity of the solutions, it is possible to use a higher flow rate and shorter needle-to-collector distance. The applied voltage, ranging from 17.4 to 18 kV, was consistent across all samples and aligns with values found in the literature for analogous systems, such as lignin-PAN or lignin-PEO blends [314,331]. These parameters ensured stable jet formation and reduced the occurrence of common defects like beads formation or fibers breakage.

The macroscopic appearance and structural consistency of lignin nanofiber mats (Lig-56%) were examined after electrospinning, as shown in Figure 45.

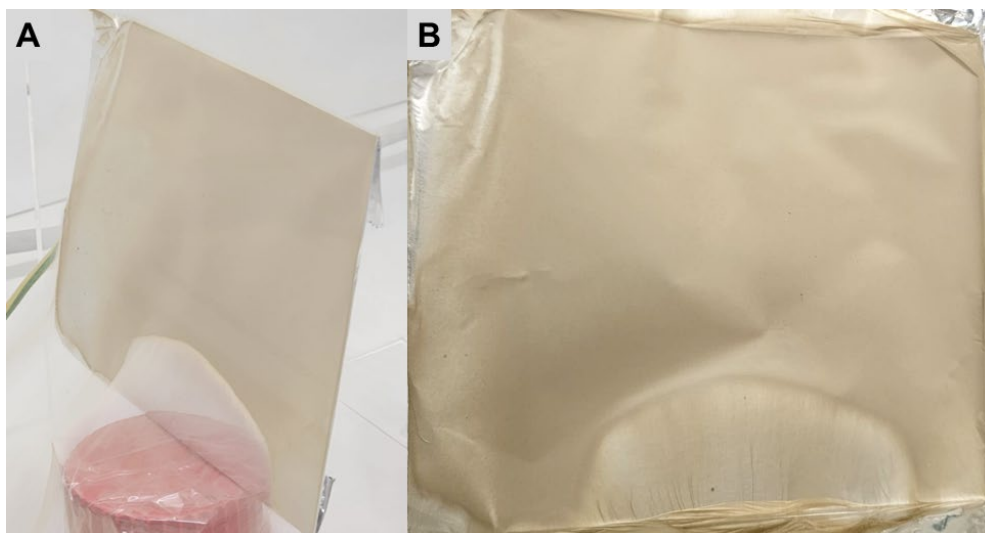


Figure 45: Macroscopic appearance of lignin nanofibers: (A) Lignin nanofiber mat (Lig-56%) deposited on the collector, captured from the electrospinning setup. (B) The same lignin nanofiber mat removed from the collector and placed on aluminum foil.

The nanofiber mats exhibited a uniform deposition on the collector and a smooth, continuous structure upon removal, with the characteristic brownish color typical of lignin-based materials, indicating the macroscopic consistency of the electrospun fibers.

4.2.3 Morphologies Nanofibers by Scanning Electron Microscopy

SEM analysis allowed the evaluation of the morphology of lignin-based nanofibers, which showed to be significantly influenced by the lignin content in the precursor material. Figure 46 shows the SEM micrographs of lignin nanofibers at two different magnifications, indicating how lignin content affects the morphology of the fibers. At lower lignin content, the fibers tend to exhibit a more uniform and aligned structure, while as the lignin content increases, the formation of thicker and more irregular fibers is observed. This behaviour can be attributed to changes in the solution's characteristics, like its viscosity, conductivity, or surface tension, which as described in Section 2.2 highly affect the final product of electrospinning.

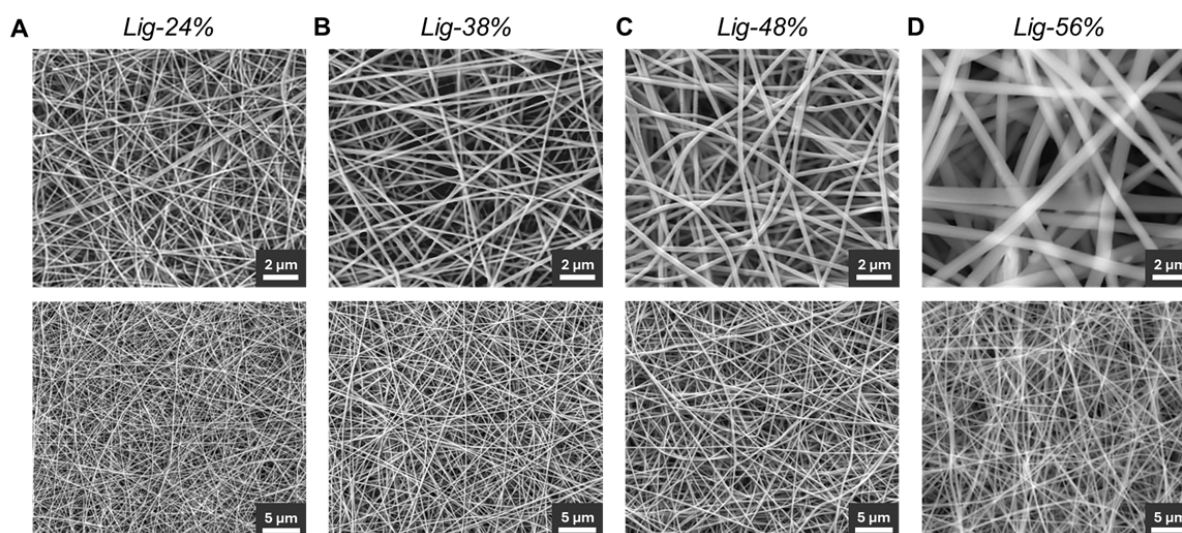


Figure 46: **Representative SEM images of electrospun fibers with different lignin content:** (A) Lig-24%, (B) Lig-38%, (C) Lig-48%, and (D) Lig-56%. The first row displays images at a magnification corresponding to a scale bar of 2 μm , while the second row shows images at a magnification corresponding to a scale bar of 5 μm .

In accordance with this, the distribution of fibers diameters is presented, highlighting the impact of varying lignin content on fiber size. In particular, the measurements show that the fibers mean diameter increases with the lignin concentration. In Figure 47A-D are reported the diameter distributions of the different nanofibers.

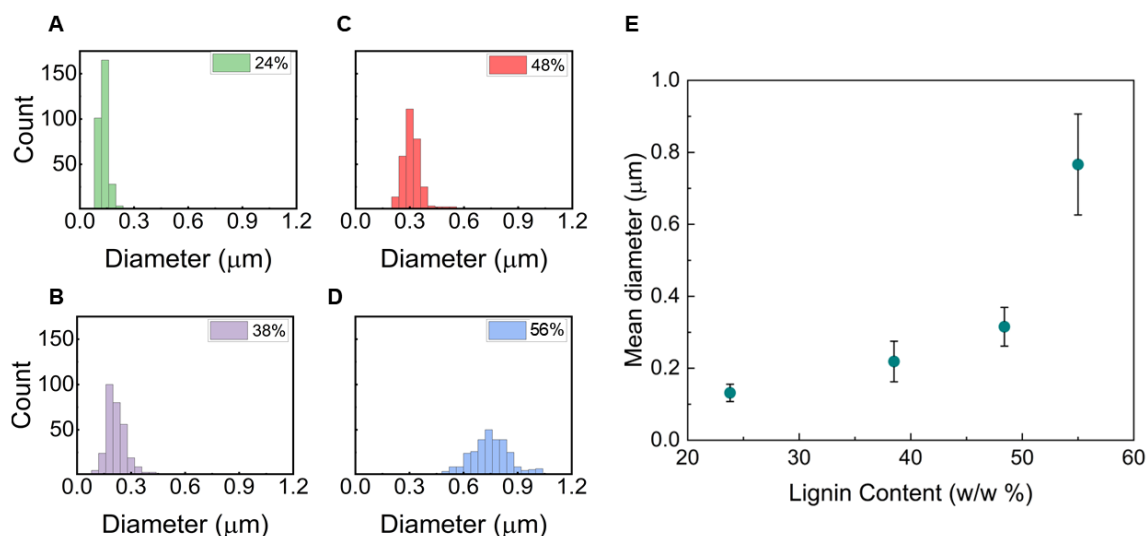


Figure 47: **Fiber diameter distribution of electrospun fibers with varying lignin concentrations:** (A) 24%, (B) 38%, (C) 48%, and (D) 56%. The histograms are based on measurements of 100 fibers from SEM images of three different samples. (E) Average fiber diameter as a function of lignin content (w/w %), with error bars represent standard deviation.

Specifically, the Lig-24% sample has an average diameter of 131.6 nm $\pm$ 24.2, the Lig-38% sample measures 218.7 nm $\pm$ 56.3, the Lig-48% sample has a diameter of 315.2 nm $\pm$ 53.8, and the Lig-56% sample shows a significantly larger average diameter of 766.2 nm $\pm$ 140.2. These results indicate that as the lignin concentration rises, the fibers diameter tends to increase, likely due to changes in solution viscosity and electrospinning dynamics (Figure 47E). Moreover, it can be observed that the sample

with the highest lignin content, 56%, exhibits the widest distribution and the largest diameters. This observation provides insight into the challenges encountered when attempting to electrospin fibers with lignin contents exceeding 56%. This threshold appears to represent a critical value beyond which spinning is likely no longer feasible, primarily due to changes in the solution properties. It would be of significant interest in future studies to explore strategies for optimizing the electrospinning process further, with the aim of increasing lignin content beyond this limit. Approaches such as varying molecular weights of PEO or employing alternative methodologies could be investigated to stabilize the process and facilitate the production of fibers with higher lignin concentrations.

4.2.4 ATR-FTIR Characterization of Lignin Nanofibers

ATR-FTIR spectroscopy was employed to analyze the molecular characteristics of the nanofibers. The spectra, recorded over the range of 400–4000 cm^{-1} , were divided into three distinct regions to facilitate a detailed analysis of the absorbance peaks. The results are reported in Figure 48 showing spectra for the regions (A) 3750–2500 cm^{-1} , (B) 1800–1200 cm^{-1} , and (C) 1200–400 cm^{-1} , respectively. Pristine lignin powder and PEO spectra are reported as reference.

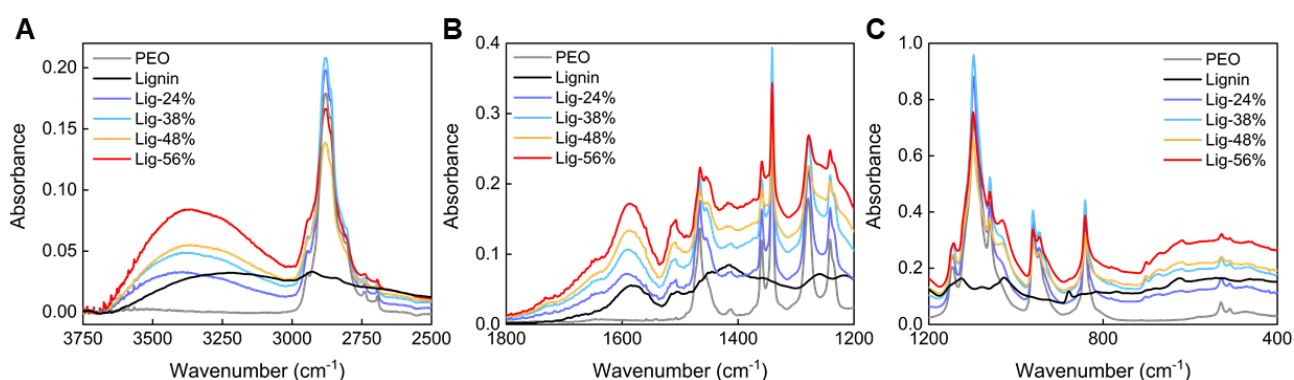


Figure 48: **ATR-FTIR spectra** of pure PEO, pristine lignin powder, and lignin-PEO nanofibers with varying lignin concentrations (24%, 38%, 48%, and 56% by weight) are presented for three spectral regions: (A) 3750–2500 cm^{-1} , (B) 1800–1200 cm^{-1} , and (C) 1200–400 cm^{-1} . Spectra of pristine lignin powder and PEO are provided as reference.

The spectra reveal key features that confirm the incorporation of lignin. In particular, in Figure 48A, the broad band detected in the range 3000–3600 cm^{-1} in the nanofibers, centered at 3300 cm^{-1} , is primarily associated with the stretching vibrations of hydroxyl groups from lignin corresponds to -OH stretching vibrations and shifts slightly with increasing lignin content, indicating changes in the hydrogen-bonding environment. This shift, compared to pure lignin, suggests interactions between lignin and PEO hydroxyl groups [332,333]. The region between 1800 and 1200 cm^{-1} showed in Figure 48B highlights vibrations associated with both lignin and PEO. For lignin, can be identified aromatic and carbonyl-related vibrations such as the prominent peak centered at 1585 cm^{-1} , associated with the aromatic C=C stretching vibrations, characteristic of lignin's phenylpropanoid structure, while the peak at 1505 cm^{-1} corresponds to aromatic ring C=C stretching vibrations of lignin [334]. Both peaks are

observed in the spectra of pure lignin and lignin-containing nanofibers.

Peaks near 1340 and 1360 cm^{-1} are attributed to C-H bending vibrations, particularly from methyl groups, while the bands at 1465 and 1450 cm^{-1} are related to CH_2 bending (scissoring) vibrations. These PEO-specific features are present in the nanofiber spectra at consistent positions, confirming the retention of PEO's structural characteristics alongside the incorporation of lignin.

The peaks at 1240 cm^{-1} and 1280 cm^{-1} correspond to C-O stretching vibrations in the ether bonds of PEO, with the latter also associated with C-H bending. [335,336]. Peaks around 1340 and 1360 cm^{-1} correspond to C-H bending vibrations, particularly from methyl groups, while the bands at 1465 and 1450 cm^{-1} are associated with CH_2 bending (scissoring) vibrations [337,338]. These PEO-related peaks are observed in the nanofiber spectra at similar positions, confirming the retention of PEO's structural characteristics alongside the incorporation of lignin.

The fingerprint region (1200–400 cm^{-1}) reveals key structural features of both components (Figure 48C). Peaks at 840 and 960 cm^{-1} , as well as shoulders at 940 and 1030 cm^{-1} , are attributed to the PEO backbone and lignin [332], respectively. The peak at 1097 cm^{-1} , with shoulders at 1058 and 1140 cm^{-1} , corresponds to C–O–C stretching in PEO [336], while new lignin-related features, such as a shoulder at 1124 cm^{-1} and a broad band between 400 and 700 cm^{-1} , out-of-plane bending of aromatic C–H bonds and phenolic hydroxyl groups of lignin [339]. This investigation revealed the successful incorporation of lignin into the nanofibers and provided evidence of distinct packing arrangements within the material.

4.2.5 Characterization of Autofluorescence in Lignin-Based Nanofibers

When evaluating the structure-related properties of lignin-nanofibers, autofluorescence emerges as a notable feature, enabling non-invasive analysis without the need for external fluorescent staining. This property originates from the aromatic rings in lignin, which are interconnected by β -O-4 ether linkages [340]. The high degree of conjugation among lignin's structural units results in a broad fluorescence emission spectrum, with excitation wavelengths spanning both the UV and visible ranges. This characteristic, referred to as multimodal fluorescence, indicates the presence of multiple fluorophores within the lignin structure. Importantly, the molecular arrangement and packing density within the lignin matrix strongly influence this property. For instance, fluorescence emission shifts to longer wavelengths in aggregated states, a phenomenon known as aggregation-induced emission (AIE). In such states, the restriction of intramolecular motion suppresses non-radiative decay pathways, leading to red-shifted fluorescence emission [341].

In this study, two-photon excitation fluorescence microscopy was employed to visualize lignin nanofibers at various concentrations. Representative 1024×1024 images, captured with two-photon

excitation at 840 nm and emission detected in the 450–650 nm range, are presented in Figure 49.

Notably, no fluorescence was detected in fibers composed solely of PEO (data not shown), confirming that the observed fluorescence signal originates exclusively from lignin.

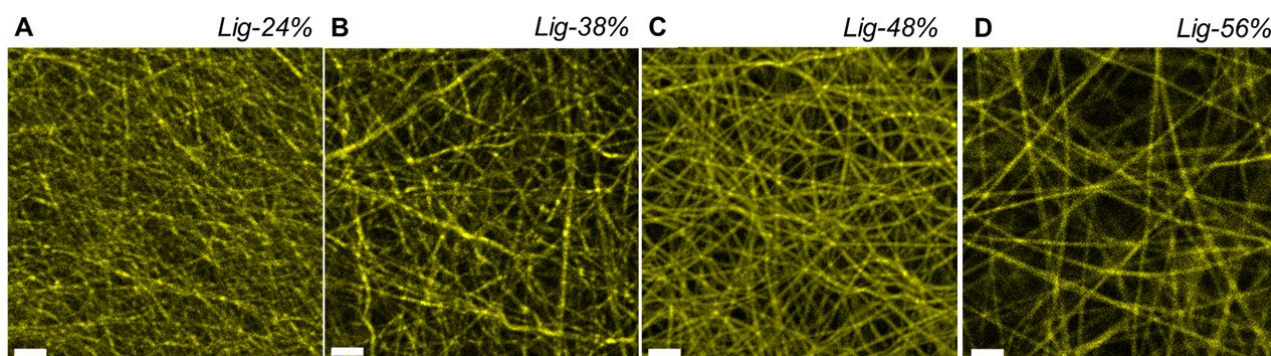


Figure 49: Representative two-photon excitation fluorescence microscopy images of electrospun fibers at varying lignin concentrations: (A) Lig-24% w/w, (B) Lig-38% w/w, (C) Lig-48% w/w, and (D) Lig-56% w/w. Lignin autofluorescence was excited at 840 nm, with emission detected in the 450–650 nm range. The images highlight the distribution and morphology of the fibers at different lignin concentrations. Scale bar: 5 μ m.

These images demonstrate the capability of two-photon fluorescence imaging to visualize the fibrous matrix across samples. At lower lignin concentrations, the fibers appear thinner and less distinct, with a denser overall arrangement. At higher lignin concentrations, the fibers exhibit larger diameters and more defined structures, consistent with observations made using SEM.

Additionally, in almost all samples, thickened regions resembling nodes are visible at the intersections of the fibers. These features likely represent localized accumulations of lignin formed during electrospinning, potentially influenced by fiber tension, solution viscosity, or phase separation effects. These nodes may also contribute to the mechanical behaviour of the fiber matrix by reinforcing interconnectivity or serving as anchoring points.

Moreover, using two-photon excitation microscopy, it was also possible to measure the emission spectra of lignin nanofibers. Emission spectra were collected from 256 $\times$ 256 pixels regions, excited at 840 nm, and detected over the 400–700 nm range (Figure 50).

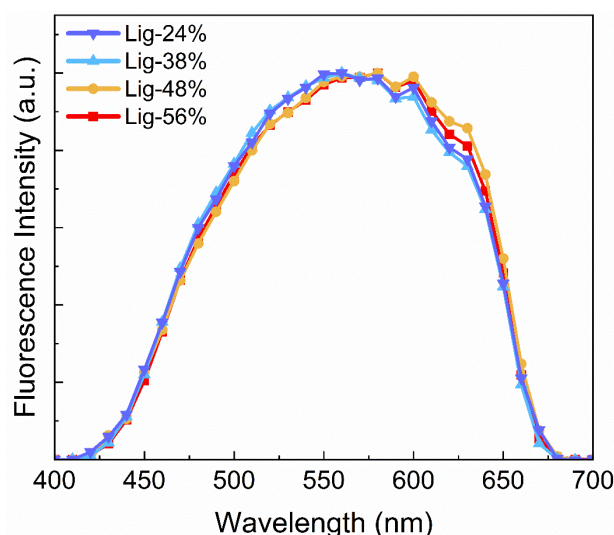


Figure 50: **Fluorescence emission spectra of electrospun fibers with varying lignin concentrations:** Lig-24%, Lig-38%, Lig-48%, and Lig-56%. The spectra represent the average signal measured at five different points on the fibers and were subsequently normalized to their respective maxima. The spectra were obtained using two-photon excitation at 840 nm, with emission acquired in the range of 400–700 nm.

The emission spectra of nanofiber samples with increasing lignin content (24%, 38%, 48%, 56%) reveal a broad fluorescence band. Given that the samples may vary in thickness and exhibit differences in density, slight band shifts can be observed within the limits of the signal-to-noise ratio, depending on the lignin concentration in the fibers. Specifically, samples with lower lignin content (24% and 38%) display peaks slightly shifted towards shorter wavelengths (blue shift), whereas samples with higher lignin content (48% and 56%) exhibit peaks shifted towards longer wavelengths (red shift). These variations may suggest the presence of two or more components contributing to the overall emission signal. Thus, it can be hypothesized that these minor shifts are attributable to differences in the intermolecular organization of lignin within the fibers, influenced by its concentration. At certain concentrations, lignin molecules may become more densely packed or differently arranged, potentially altering their emission properties.

The autofluorescence of lignin enables not only 2D imaging but also the investigation of the three-dimensional (3D) arrangement of the nanofiber matrix. Visualizing the matrix in 3D is particularly significant as it offers in-depth understanding of the spatial distribution and organization of lignin within the fibers, thereby offering a comprehensive understanding of the internal architecture, continuity, and potential heterogeneities present in the material.

As an illustrative example, Figure 51 presents a three-dimensional reconstruction of 20 sections images along z-axis, of a sample of lignin nanofiber with the highest lignin content (Lig-56%), highlighting the spatial arrangement and interconnected structure of the fibers.

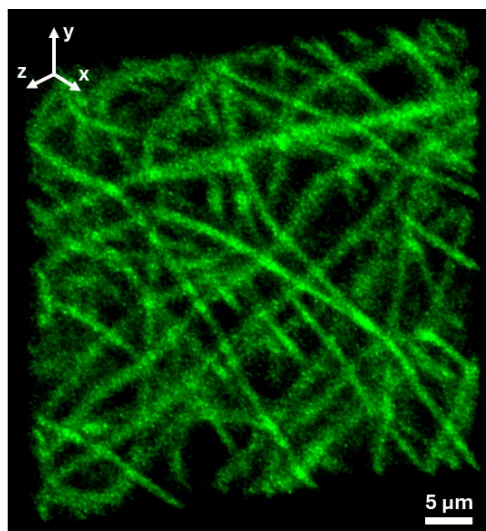


Figure 51: **Three-dimensional visualization of electrospun lignin nanofibers (Lig-56%)** obtained through two-photon excitation microscopy. The image was generated by stacking 20 multiple optical sections acquired along the z-axis. The autofluorescence of lignin was excited at 840 nm, and emission was collected in the range of 400–700 nm. The signal corresponds to the intrinsic emission of lignin, allowing the visualization of the fibrous matrix. The scale bar is 5 μm .

The image reveals a uniform distribution of lignin throughout the three-dimensional structure, suggesting consistent integration within the fibrous matrix. A highly entangled network with well-defined fibers, which appear continuous across the imaged volume, is discernible. This result underscores the potential of utilizing the intrinsic autofluorescence of lignin for non-invasive investigations of nanofiber structures without the need for external dyes or labels, thus preserving the native state of the material. This capability is critical for studying the interaction of fibers with biological environments or monitoring their behaviour over time under physiological conditions.

3D visualization also provides a means to explore how structural features, such as fiber density and continuity, might influence key biomedical factors, including cell adhesion, proliferation, or other relevant parameters.

To further analyze the photophysical properties of lignin, fluorescence lifetime imaging microscopy (FLIM) was employed following fluorescence spectral analysis and 2D/3D imaging. FLIM measurements were performed on the four nanofiber samples with increasing lignin content (Lig-24%, Lig-38%, Lig-48%, Lig-56%). To clearly illustrate the differences in fluorescence lifetime distributions, data are presented exclusively for the samples with the highest (Lig-56%) and lowest (Lig-24%) lignin concentrations, as these showed the most significant differences, visualized through the phasor plot. Representative 256 x 256 pixels intensity maps are presented in Figure 52A, showing the spatial distribution of the fluorescence signal across the fibers matrix. The associated phasor maps are shown in Figure 52B, where the color of each pixel in the intensity maps corresponds to the phasor plot [156] (Figure 52C). Notably, the phasor plot reveals two distinct distributions of fluorescence lifetime, marked by circular cursors in green and red, providing insights into the fluorescence lifetime

of lignin-based nanofibers and indicating that the sample Lig-56% exhibits shorter average fluorescence lifetimes compared to the sample Lig-24%.

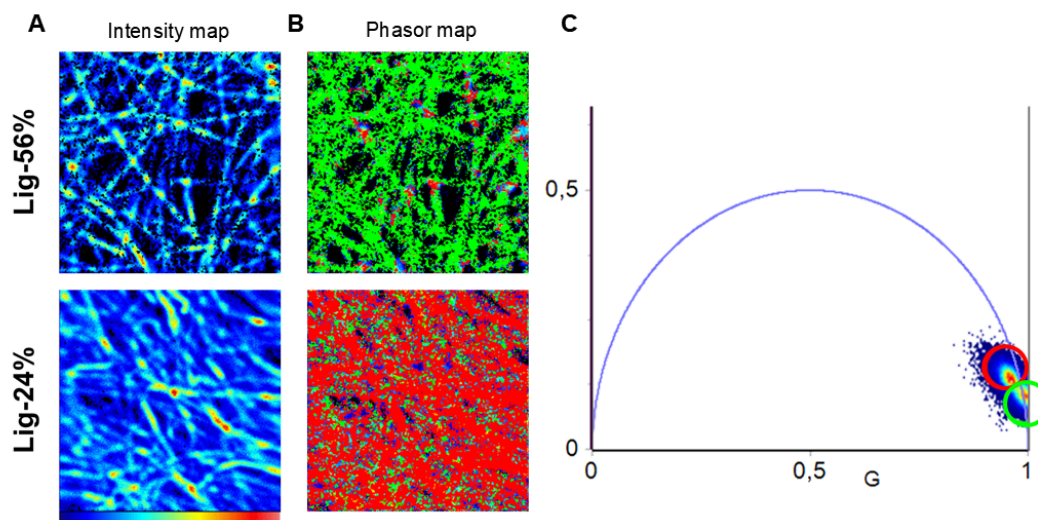


Figure 52: **FLIM analysis of electrospun nanofibers with varying lignin concentrations:** (A) Intensity maps (256 x 256 pixels) of Lig-56% (top) and Lig-24% (bottom). (B) Phasor lifetime maps for Lig-56% (top) and Lig-24% (bottom), with each pixel assigned a color according to its position in the phasor plot (C). The phasor plot highlights two distinct lifetime distributions, indicated by the green and red circular cursors. The two samples are characterised by distinguishable lifetime being the one at higher concentration shorter (green cursor) representing Lig-56%, while the sample at lower concentration, Lig-24%, with a longer lifetime (red cursor).

The difference in lifetime distributions is in line with observations from the fluorescence emission spectra, where two distinct spectral components were identified. These two behaviours can be rationalized by considering the distinct molecular environments of lignin in the two samples.

In the Lig-56% sample, the shorter fluorescence lifetime may result from a higher degree of lignin packing within the nanofiber matrix. This observation is consistent with studies on the fluorescence lifetime of lignin in wood and lignocellulosic biomass, where more compact or aggregated lignin structures exhibit significantly shorter lifetimes, typically in the range of 0.5–1 ns, due to enhanced heterogeneity and structural disorder that accelerate non-radiative decay pathways. While less densely packed lignin structures exhibit longer fluorescence lifetimes, typically around 4 ns [342]. For instance, Donaldson et al. reported fluorescence lifetimes of 0.418 ns for lignin in compressed wood compared to 0.473 ns in normal wood, underscoring the importance of packing density and structural integrity in determining the fluorescence properties of lignin. Similar mechanisms may govern the shorter fluorescence lifetimes observed in the Lig-56% sample [343].

Interestingly, in the Lig-56% sample, particular nodes at the intersections of the fibers can be observed as previously described, which exhibit longer fluorescence lifetimes compared to the surrounding fibers. This observation may indicate that, in these regions, lignin organizes differently within the nanofiber matrix. Alternatively, these nodes could correspond to areas where PEO is more concentrated, resulting in altered fluorescence lifetime characteristics.

Conversely, in the Lig-24% sample, the lower lignin content results in a less dense molecular arrangement. This reduced packing diminishes intermolecular interactions, leading to longer fluorescence lifetimes. These observations, consistent with the shifts observed in the emission spectra, suggest that the distinct lifetime distributions arise from differences in lignin packing, supporting the hypothesis that the two lifetime components correspond to the two emission spectral components observed.

In conclusion, fluorescence lifetime imaging microscopy (FLIM) provides a powerful tool for characterizing the photophysical properties and molecular organization of lignin-based nanofibers. Further studies could explore the correlation between fluorescence lifetime measurements and the mechanical properties of the nanofibers. Understanding how molecular packing, lignin concentration, and spatial organization influence both fluorescence lifetimes and mechanical behaviour would provide critical insights for tailoring nanofiber materials for specific applications. For instance, regions with distinct lifetimes, such as the fiber intersections or nodes observed in Lig-56%, may play a role in the mechanical strength, elasticity, or other functional properties of the material. Moreover, FLIM has significant potential as a rapid and high-throughput method for material characterization. Its ability to gain comprehensive, non-invasive information about molecular interactions and organization makes it a promising technique for screening and differentiating between various nanofiber systems [344].

Following these characterizations of lignin-based nanofibers, the following sections aim to expand on their functional characteristics and potential applications. The intrinsic properties of lignin, such as its mechanical properties, antioxidant activity, and biocompatibility, are inherently tied to its molecular structure and organization within the nanofibers. These relationships underscore the critical interplay between structure and function in determining the performance and versatility of the material. By exploring these interconnections, the following sections aim to provide a comprehensive understanding of how the unique composition and architecture of lignin-based nanofibers contribute to their multifunctionality.

4.2.6 Mechanical Properties of Lignin Nanofibers

A fundamental aspect of lignin nanofibers as a biomaterial is the study of their mechanical characteristics, as these are critical in determining their performance in both structural and functional applications. Understanding the relationship between structure and function is fundamental to optimizing the nanofiber matrix for specific uses. This is particularly relevant in designing biomaterials, where mechanical performance directly influences their interaction with biological tissues and their behaviour under physiological loads. For instance, the material must exhibit suitable mechanical properties to conform effectively to body surfaces, a critical requirement for applications

such as wound dressings or bandages [104]. DMA was employed to evaluate these properties, enabling precise measurements of the stress-strain behaviour of the nanofibers. The stress-strain curves for nanofibers are presented in Figure 53, revealing noticeable differences in mechanical behaviour across the samples.

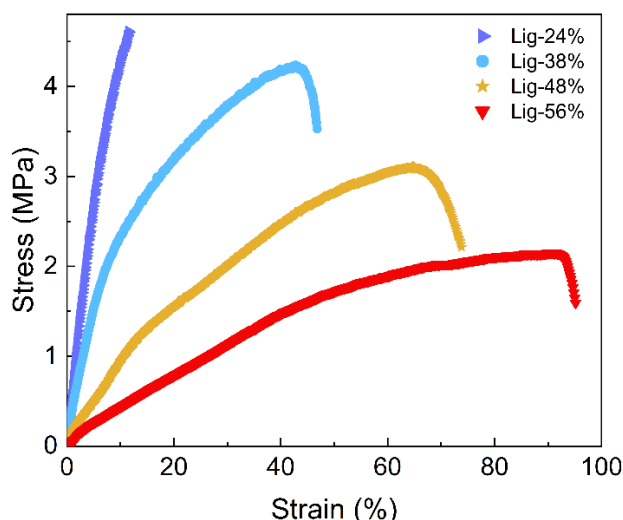


Figure 53: **Stress-strain curves of lignin-based fibers with different lignin contents: 24%, 38%, 48%, and 56% w/w %**, showing stress (MPa) as a function of strain (%).

The curves demonstrate a transition as the lignin content increases, with steeper slopes observed for samples with lower lignin content, suggesting greater stiffness. In contrast, the slopes become progressively decrease as the lignin content rises, indicating a reduction in rigidity and a shift towards a more flexible mechanical profile.

Moreover, to quantify this change in the mechanical behaviour, the Young's modulus values were calculated from the linear elastic region of the stress-strain curves and are reported in Table 2, showing a dependency on the lignin content within the nanofibers.

Sample	Young's Modulus (MPa)
Lig-24%	36 ± 3
Lig-38%	29 ± 4
Lig-48%	9.7 ± 1.3
Lig-56%	3.9 ± 0.4

Table 2: **Young's modulus (MPa) values for lignin-based nanofibers with varying lignin contents.**

It is possible to observe an inverse relationship between Young's modulus and lignin content, with stiffness progressively decreasing as lignin concentration increases. This trend suggests that higher lignin concentrations contribute to a more flexible nanofiber matrix. PEO likely plays a key role in enhancing flexibility, as its proportion increases relative to lignin at lower concentrations. Furthermore, supporting this hypothesis, previous studies indicate that lignin can act as a plasticizer by disrupting interpolymer interactions with the crystalline regions of PEO, increasing the free volume between polymer chains, and enabling better molecular alignment and tighter fiber packing [345,346]. Similar behaviours have been observed in the literature; for instance, the incorporation of kraft lignin into PLA matrices has demonstrated that higher lignin content reduces tensile strength, thereby decreasing rigidity [347]. Our results thus attribute to the lignin content the ability to modulate the mechanical properties of the nanofibers, which presents an interesting advantage that can be tuned based on the desired final application of the biomaterial. For instance, more flexible nanofibers may be desirable for wound dressing applications, as demonstrated in studies on lignin-cellulose composites with antibacterial and antioxidant properties [348]. Conversely, stiffer nanofibers can be employed in applications such as bone scaffolds, where lignin enhances hydrophilicity and mechanical stability [349].

4.2.7 Antioxidant Properties of Lignin Nanofibers

The antioxidant properties of the nanofibers represent another critical aspect in the evaluation of their functional characteristics. Following the analysis of their mechanical behaviour, the focus now shifts to the intrinsic antioxidant capacity of lignin, and in particular, to its ability to retain these properties even once incorporated into the nanofibers.

Lignin's ability to scavenge free radicals [350,351] is mainly associated with the hydroxyl and methoxy groups in its structure, enabling to neutralize radicals by donating hydrogen atoms, thereby reducing oxidative stress [352]. To evaluate the retention of this property in nanofiber formulations, the antioxidant activity of lignin nanofibers (Lig-NFs) was tested by the DPPH assay (2,2-diphenyl-1-picrylhydrazyl). Results for different lignin concentrations (24%, 38%, 48%, and 56%) over time are presented in Figure 54. The explanation of the assay's principles is provided in Section 4.4.

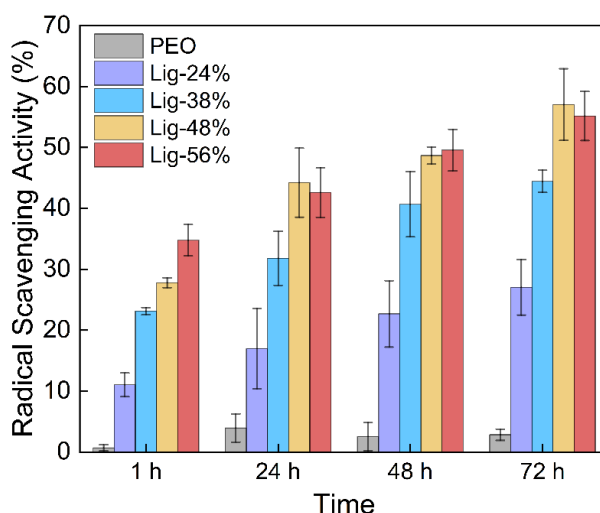


Figure 54: **Radical scavenging activity (RSA%) of nanofibers with varying lignin contents (24%, 38%, 48%, and 56%) and PEO control over time (1 h, 24 h, 48 h, and 72 h), measured using the DPPH assay. Error bars represent standard deviations ($n = 3$).**

It is possible to observe that after 1 hour, nanofibers containing 24%, 38%, 48%, and 56% lignin exhibited Radical Scavenging Activity (RSA%) values of approximately 23%, 28%, 35%, and 35%, respectively. Over time, these values increased, with the 56% lignin nanofibers reaching an RSA% of approximately 55% at 72 hours. This trend indicates that the antioxidant activity of lignin persists even after the electrospinning process of the solution and its subsequent release from the nanofibers.

Comparisons with literature data on lignin's antioxidant activities are challenging due to variability in how lignin content is expressed across different materials. However, the mean RSA% of 55% at 72 hours observed in this study is consistent than the reported average for similar systems [309,353,354]. Importantly, the primary aim of this work was to determine whether lignin retained its antioxidant activity when encapsulated within nanofibers. These findings indicate that RSA% continues to increase even after three days, suggesting that lignin maintains its scavenging activity when incorporated into the PEO blend. PEO, used as the control material, exhibited negligible antioxidant activity across all time points, confirming that lignin is the principal contributor to the observed radical scavenging properties.

To further contextualize these findings, RSA% at 24 hours was compared with lignin in its pure form (Lig) and ascorbic acid (AA) as a positive control (Figure 55). Pure lignin displayed a high RSA% at 24 hours, which reflects the immediate accessibility of its active functional groups in the unstructured form. Ascorbic acid similarly achieved 100% RSA at 24 hours, consistent with its rapid and complete scavenging capacity, commonly observed in literature. These controls serve as important benchmarks: ascorbic acid establishes the upper theoretical limit for scavenging activity, while pure lignin reflects the maximum activity achievable by lignin molecules in the absence of structural modifications.

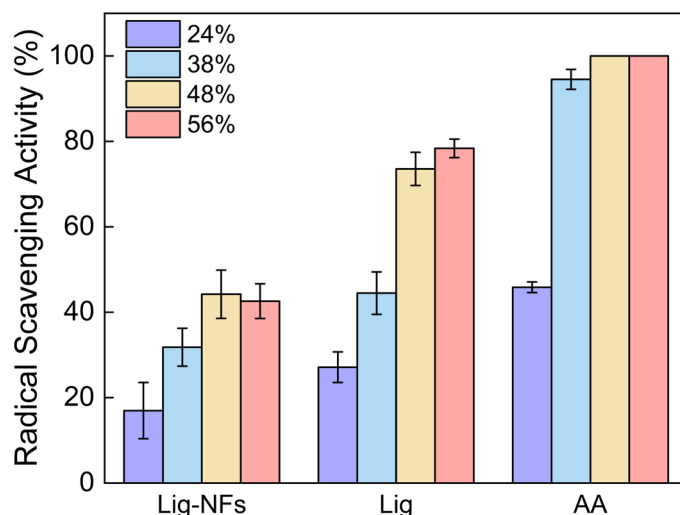


Figure 55: Antioxidant activity (% Radical Scavenging Activity (RSA) at 24 hours for lignin nanofibers (Lig-NFs), pure lignin (Lig), and ascorbic acid (AA) as the positive control. Lignin nanofibers were tested at four lignin concentrations (24%, 38%, 48%, and 56%), while pure lignin and ascorbic acid were evaluated at the corresponding concentrations.

The lower RSA% observed for lignin nanofibers compared to pure lignin at 24 hours suggests that the structural configuration of lignin within the nanofiber matrix may initially influence its reactivity. Factors such as molecular arrangement, physical entanglement with PEO, or possible steric hindrance could limit the immediate availability of phenolic and methoxy groups for radical neutralization. However, the increase in RSA% over time indicates that these groups remain active and continue to participate in the scavenging process as they gradually interact with the radicals in solution. This prolonged scavenging activity, highlights a kinetic behaviour where lignin's antioxidant potential is sustained over time, possibly due to the delayed interaction of its functional groups with DPPH radicals. Such sustained activity is particularly advantageous for biomedical applications, such as wound healing, where the continuous presence of antioxidant compounds helps mitigating oxidative stress and promotes tissue regeneration [355,356]. In this regard, Xu et al. proved that the incorporation of lignin accelerated significantly the healing of chronic wounds in mice, thanks to its antioxidant activity during time [357].

4.2.8 Evaluation of Nanofiber Biocompatibility

Biocompatibility is a fundamental requirement for biomaterials intended for medical applications. To evaluate this property, we conducted tests on human fibroblast cells exposed to nanofibers composed of lignin at maximum concentration and compared the results to those obtained from nanofibers made solely of PEO. The outcomes, illustrated in Figure 56, indicate that cell viability remains around 80% after exposure to lignin nanofibers, which is within the acceptable range for biocompatibility

assessments. As a biomaterial is considered biocompatible if it does not cause more than a 30% reduction in cell viability, corresponding to a viability threshold of 70% [358,359].

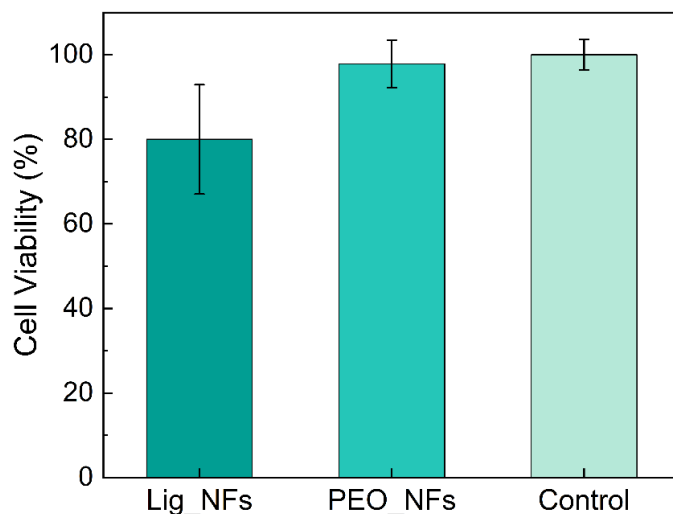


Figure 56: **Cell viability of human fibroblasts cultured on electrospun fibers.** The bar graph shows the percentage of viable cells for different materials: Lig_NFs (lignin-based nanofibers at 56% w/w content of lignin), PEO_NFs (PEO-based nanofibers), and the control group. Cell viability was measured after 4 hours of incubation. Error bars represent standard deviation.

The slight decrease in cell viability observed with lignin nanofibers could be due to the alkaline pH employed or the chemical composition of lignin, which contains a high concentration of phenolic and aromatic groups. These functional groups can interact with cellular components, potentially leading to mild membrane perturbations. Additionally, the physical characteristics of lignin nanofibers, such as surface roughness might influence cell adhesion and proliferation differently compared to PEO nanofibers, which are known for their excellent biocompatibility [360]. Despite this modest reduction in viability, lignin nanofibers demonstrate promising potential for biomedical applications.

4.2.9 Aqueous Dissolution Properties of Lignin Nanofibers

The dissolution properties of lignin nanofibers were investigated to assess their behaviour in aqueous environments, given that they are fabricated through waterborne electrospinning. Understanding how these nanofibers interact with water is crucial for determining their stability and identifying suitable applications. The ability of the fibers to dissolve can dictate their potential use in fields such as drug delivery, tissue engineering, or other biomedical and environmental applications. This study focused on samples with 56% lignin content (Lig-56%) due to their higher lignin concentration, which makes them representative of the matrix's dissolution behaviour and facilitates a direct analysis of lignin's release profile. The study was conducted by immersing the nanofibers samples in water and monitoring the time required for complete disintegration. The dissolution process was characterized by a rapid dissolution as shown in Figure 57, until complete disappearance of the disks within approximately 15

minutes. This rapid dissolution is attributed to the high wettability of the nanofibrous matrix, allowing water to penetrate and disintegrate the structure efficiently. These findings align with the classification of these materials as fast-dissolving nanofibers (FDNs) [361–364], releasing lignin into the aqueous medium in a short-period.

Figure 57 presents images extracted from frames of a video capturing the immersion process of a disk weighing approximately 5 mg, composed of Lig-56% nanofibers, into water over time.

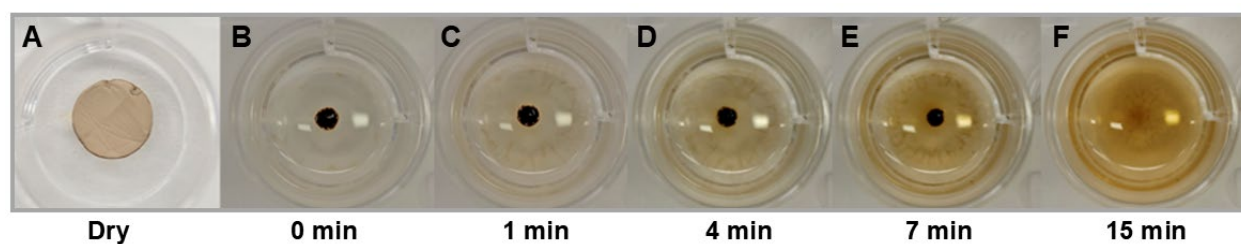


Figure 57: Sequential photographs of lignin nanofibers with the highest lignin content (Lig-56%) during dissolution in water, showing the dry state (A) and time points at 0 (B), 1 (C), 4 (D), 7 (E), and 15 minutes (F). The images were extracted from a video recorded to monitor the dissolution process, initiated by the immersion of a 5 mg nanofiber disc in 100 μ l of water and continuing until complete disintegration of the sample.

As shown in the sequential photographs, the disc rapidly dissolves upon immersion, accompanied by a progressive darkening of the solution. This observation indicates that lignin is being dispersed into the aqueous medium, leading to a reduction in the nanofiber structure and increased release of lignin into the fluid. The change in color provides qualitative evidence of the dissolution process, while concentration measurements confirm the quantitative release of lignin over time. This behaviour highlights the high wettability and rapid dispersion properties of the nanofibers, which make them ideal for applications that demand rapid lignin release.

In another study, we aimed to investigate the corresponding increase in lignin concentration in solution over time. The release of lignin into the water followed a gradual release throughout the dissolution period, reaching a full release at 30 minutes (Figure 58).

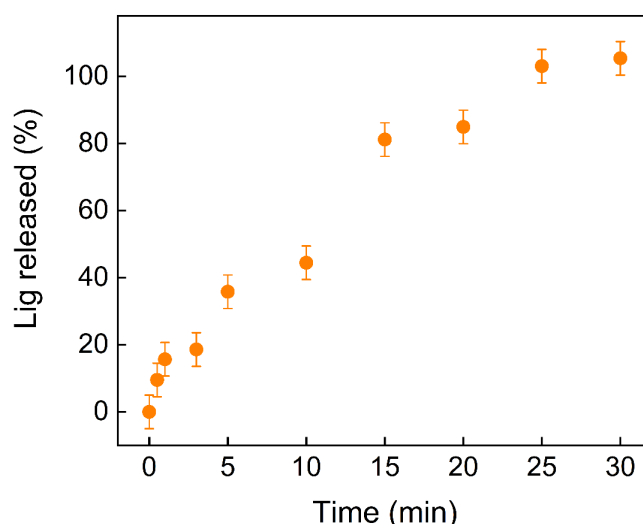


Figure 58: **Release profile of lignin from nanofibers with the highest lignin content (Lig-56%) as a function of time during dissolution in water.** The percentage of lignin released (% Lig released) was determined at specific time points over a 30-minute period, calculated relative to the theoretical lignin content of the disc. The lignin concentration was monitored spectrophotometrically at 280 nm. The results demonstrate a rapid initial release of lignin, followed by a slower release phase that approaches complete dissolution by 30 minutes.

As the matrix progressively disintegrates, the lignin contained within is gradually released into the solution, ultimately leading to the complete solubilization of its content. The dissolution process likely reflects the progressive exposure of different regions of the nanofiber matrix to water over time. This variation in accessibility may influence the release dynamics, with the exposed regions dissolving first, while less accessible portions require more time to disintegrate. Over time, the rate of release appears to slow, eventually reaching 100% dissolution by approximately 30 minutes. These findings suggest several promising research directions beyond the scope of this thesis. For example, these nanofibers could be explored as DDS, offering rapid release and enhanced bioavailability. Pilot experiments have suggested that the release profile can be modulated based on how lignin is packed within the nanofiber matrix, highlighting the potential for tailoring the dissolution behaviour. This tunability, influenced by variations in nanofiber structure and composition, expands their potential applications, such as using thinner nanofibers for immediate release and thicker ones for sustained or delayed release systems. These results form the foundation for exploring lignin nanofibers as a carrier for active molecules. The rapid dissolution of the fibers in aqueous environments makes them an excellent candidate for applications requiring efficient and controlled substance release, as demonstrated in the following section with the incorporation of a model antibacterial drug.

4.2.10 Proof-of-Concept: Antibacterial Drug Incorporation and Activity in Lignin Nanofibers

Given the rapid dissolution properties of lignin nanofibers, their potential as DDS was further investigated by evaluating their ability to encapsulate drugs while maintaining morphological integrity and ensuring a stable electrospinning process. To this end, bacitracin, a polypeptide antibiotic derived

from *Bacillus subtilis*, was selected as a model drug due to its efficacy against Gram-positive bacteria and its moderate aqueous solubility which presents challenges in topical antibacterial delivery systems [365–367].

In this thesis, the electrospinning process was successfully optimized to fabricate stable and uniform fibers, achieving effective encapsulation of bacitracin while maintaining the desired morphological properties.

To achieve this, the electrospinning process was modified by adding aqueous bacitracin solutions to lignin dispersions. The initial lignin dispersion was prepared at a concentration of 10% w/w prior to mixing with bacitracin and PEO. This approach resulted in fibers with a final composition of 51% lignin, 40% PEO and 9% bacitracin. SEM analysis confirmed the successful fabrication of bacitracin-loaded lignin fibers (Lig-Bac), demonstrating uniform morphology and structural integrity as shown in Figure 59.

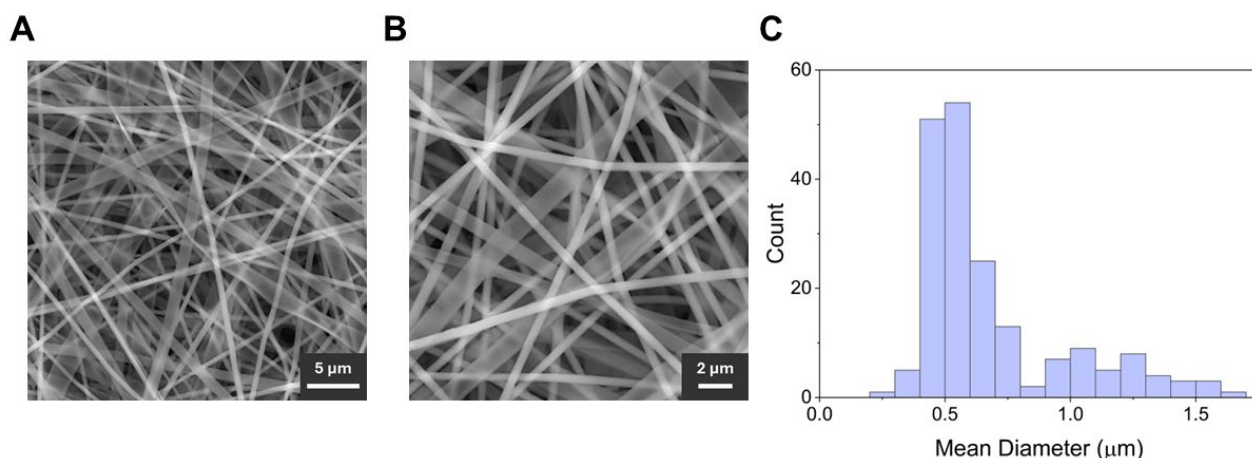


Figure 59: Representative SEM images of lignin/PEO electrospun fibers loaded with Bacitracin 9% w/w: (A) magnification corresponding to a scale bar of 5 μm, (B) magnification corresponding to a scale bar of 2 μm. (C) Histogram showing the distribution of mean fiber diameters.

It is possible to observe that the incorporation of bacitracin results in fibers with a more heterogeneous morphology compared to unloaded fibers (Lig-56%, showed in Figure 47). Notably, the loaded fibers exhibit a broader distribution, with a small fraction of fibers exceeding 1 μm in diameter. However, this result underscores the ability of the electrospinning process to encapsulate the drug within the fiber matrix effectively.

Evidence of bacitracin incorporation was obtained through micro-FTIR spectroscopy, allowing for the spatially resolved analysis of heterogeneous samples. This method was specifically applied to investigate the amide region of the IR spectra, where characteristic absorption bands are observed (Figure 60).

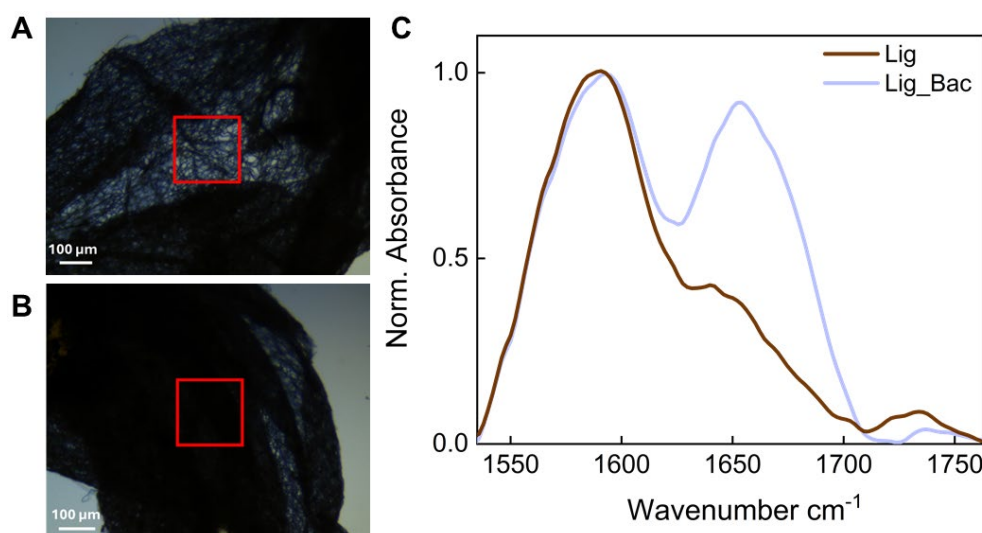


Figure 60: Micro-FTIR Characterization. Representative optical microscopy images of lignin nanofibers (A) and lignin nanofibers loaded with bacitracin (B). The red squares indicate the regions of interest (ROIs) where FTIR spectra were collected (200 × 200 μm). Scale bar is 100 μm. (C) FTIR spectra of lignin nanofibers (Lig) and lignin nanofibers loaded with bacitracin (Lig_Bac), recorded in the amide region (1550–1750 cm⁻¹). The characteristic amide II band at 1650 cm⁻¹, associated with N-H bending and C-N stretching vibrations of polypeptides, is prominently observed in the spectrum of Lig_Bac, confirming the incorporation of bacitracin within the nanofibers. The spectra were obtained from solid nanofiber samples, with the sample thickness standardized using a 15 μm spacer to ensure a consistent path length.

The optical images of lignin nanofibers (Figure 60A) and lignin nanofibers loaded with bacitracin (Figure 60B) illustrate the morphology of the samples. The red-highlighted ROIs, measuring 200 × 200 μm, indicate the areas where the micro-FTIR spectra were collected, ensuring a precise and localized analysis.

The FTIR spectra (Figure 60C) confirm the incorporation of bacitracin through the detection of the characteristic amide II band at 1650 cm⁻¹, which corresponds to N-H bending and C-N stretching vibrations. This band, a distinctive feature of bacitracin as a polypeptide antibiotic, appears with higher intensity in the lignin-bacitracin composite (Lig_Bac) compared to the lignin-only nanofibers (Lig).

As a proof of concept, an antibacterial assay was performed against *Kocuria rhizophila* (Gram-positive), evaluating the efficacy of bacitracin-loaded lignin nanofibers (Lig_Bac 9%) in comparison to unloaded lignin nanofibers (Lig), a bacitracin solution (Bac 9%), and PEO nanofibers (Figure 61). After incubation on agar plates seeded with the bacterial culture, a clear inhibition zone was observed around the bacitracin-loaded lignin nanofiber samples after 24 hours (not shown) and 48 hours, demonstrating the antibacterial properties of the Lig_Bac material. In contrast, no inhibition zone was observed for the unloaded lignin nanofibers (Lig) or the PEO nanofiber disks, indicating that neither material exhibits intrinsic antibacterial activity. Notably, the assay, conducted after 48 hours of contact with the fibers, showed no regrowth of bacteria, indicating that the antibacterial effect of Lig_Bac is bactericidal. This prolonged inhibition suggests the potential effectiveness of the material even against

dormant or persistent bacterial subpopulations, although specific assays would be required to confirm activity against persister cells.

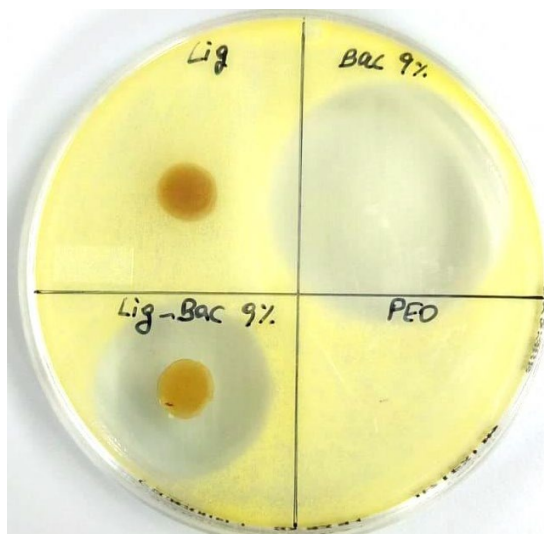


Figure 61: **Antibacterial assay against *Kocuria rhizophila*** using lignin nanofibers (Lig), bacitracin-loaded lignin nanofibers (Lig_Bac 9%), bacitracin solution (Bac 9%), and PEO nanofibers. The image shows the inhibition zones formed around the fiber samples and solution on an agar plate after 48 hours of incubation.

When comparing the inhibition zone between the bacitracin solution control and the Lig_Bac nanofiber samples, a noticeable discrepancy in zone size was observed. This difference can likely be attributed to multiple factors. First, the control is based on the nominal theoretical concentration of bacitracin in the nanofibers, as accurate determination of drug loading was not feasible despite repeated attempts using various spectroscopic techniques and quantitative assays. Second, bacitracin in solution is immediately available and acts promptly, while bacitracin from the nanofibers is released gradually over time, leading to a slower onset of antibacterial activity.

Interestingly, both the Lig and Lig_Bac 9% nanofiber disks remained visible on the agar plate after the assay. This observation suggests that the fibers retain their structural integrity during the test and do not fully dissolve or disintegrate under these conditions. This property highlights the potential durability and stability of lignin-based nanofibers in applications requiring sustained antibacterial activity.

These findings provide a proof-of-concept for incorporating bacitracin into lignin nanofibers and highlight the potential of such systems in enhancing antibacterial activity. Future applications could build on this work by exploring the incorporation of other types of drugs, potentially addressing broader therapeutic targets. Adjustments to drug concentration and molecular properties, along with refined fibers fabrication processes, could enhance both drug loading efficiency and release profiles. By tailoring the approach to the specific properties of different drugs, lignin nanofibers could serve as

versatile carriers for a wide range of pharmaceutical applications, further expanding their potential in biomedical and antimicrobial fields.

4.3 Conclusions

In this chapter, lignin-based were successfully produced using a sustainable and green approach that employs water as the sole solvent. This work was carried out with the goal of valorising lignin, a biomaterial with many interesting properties for biomedical applications that is often regarded as waste due to its challenging processability. The methodology combines strategies to enhance lignin's solubility in water with a minimalist process that relies on pH adjustment and ultrasonication to improve lignin dispersibility. By blending lignin with PEO to facilitate electrospinning, fibers with varying lignin contents, up to 56 w/w%, were fabricated. These nanofibers exhibited differences in mechanical properties and molecular organization depending on lignin concentration. This versatility extends to their potential applications, as the nanofibers can function as antioxidant systems or as matrices for the encapsulation of various molecules, enabling their controlled release. The mechanical properties of the fibers were modulated by the lignin content, with higher lignin concentrations resulting in reduced rigidity. FLIM provided insights into the molecular packing of lignin within the fibers, revealing differences between densely packed and less concentrated lignin regions. This highlights the potential of FLIM as a rapid screening tool for evaluating the internal structure of lignin-based systems. The fibers demonstrated biocompatibility, suggesting their suitability for biomedical applications. Moreover, they were capable of encapsulating bioactive molecules, such as the antibacterial drug bacitracin. As a proof of concept, bacitracin-loaded lignin nanofibers were shown to retain their antibacterial efficacy, underscoring their potential as DDSs. Beyond drug delivery, the intrinsic antioxidant properties of lignin, combined with the ability of the fibers to release lignin gradually, suggest their use in applications requiring sustained antioxidant activity.

4.4 Experimental Methodology

Materials

Alkali lignin (low sulfonate content), Poly(ethylene oxide) (PEO, Mw 900 kDa), bacitracin, NaOH, HCl, DPPH, ascorbic acid, and Bacitracin from *Bacillus licheniformis* were acquired from Sigma-Aldrich (St. Louis, MO, USA). Ultrapure Milli-Q water (18.2 M Ω , Thermo Fisher Scientific) was used for all solutions.

Evaluation of Lignin Solubility in Water

Aqueous dispersions of alkali lignin were prepared at varying w/w% concentrations. Magnetic stirring was applied for up to 2 days to ensure thorough dissolution and mixing. Aliquots (1 ml) were periodically withdrawn and centrifuged at 10000 rpm, 10 minutes. The dissolved lignin concentration ($\epsilon = 22.1 \text{ g}^{-1}\text{L cm}^{-1}$ $\lambda = 280 \text{ nm}$ [368]) was measured from the supernatant, using a Nanodrop spectrophotometer (ThermoFisher, NanoDrop 2000). pH adjustments were made with NaOH or HCl and monitored using a pH meter (Radiometer Analytical, MeterLab PHM240). Ultrasonication was performed using a Misonix S-4000-010 Sonicator (600 W, 220 V) (Bioventus LLC, Durham, NC, USA) at 60% amplitude for 30 minutes in an ice bath (10s on/7 s off), to minimize thermal degradation.

Lignin Nanofibers Fabrication by Electrospinning

The preparation process, illustrated in *Figure 62*, involved multiple steps to ensure optimal nanofiber formation.

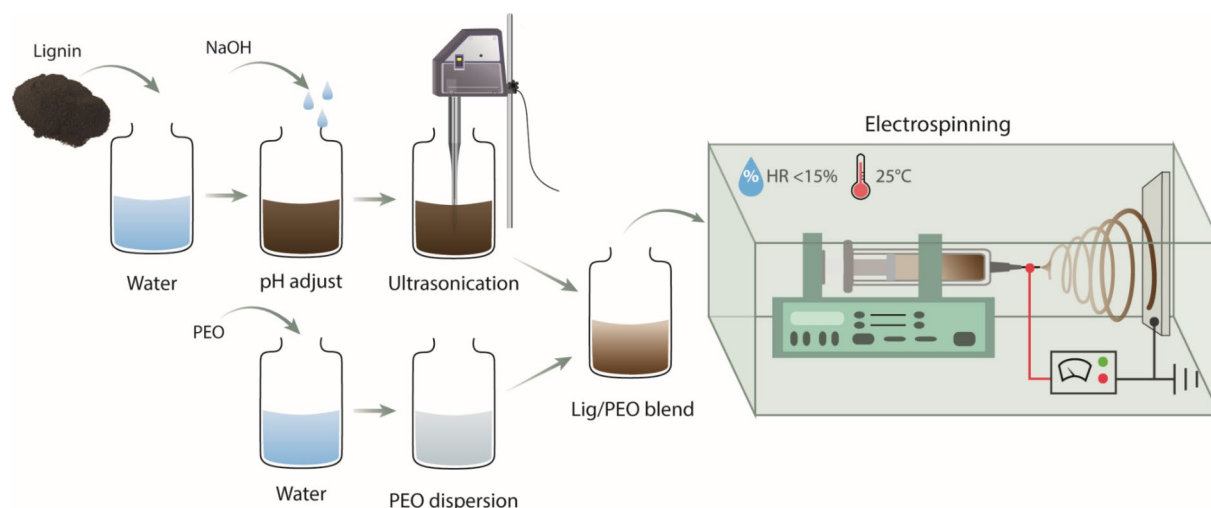


Figure 62: Illustration of the protocol developed for the preparation of lignin/PEO nanofibers via electrospinning. Lignin is dispersed in water and adjusted to an alkaline pH using NaOH, followed by ultrasonication. Simultaneously, PEO is dissolved in water to obtain a homogeneous solution. The two solutions are blended in desired ratios to prepare the lignin/PEO mixture. The blend is then electrospun in a controlled environment with controlled relative humidity (RH) and temperature to facilitate water evaporation and the effective formation of nanofibers.

Initially, lignin dispersions were stirred for two days after being adjusted to pH 11 using NaOH, which enhanced both solubility and homogeneity. Simultaneously, PEO was dissolved in water through a similar two-day stirring process. Ultrasonication was applied to the lignin dispersions to break down aggregates and improve dispersion quality before blending them with the PEO solution at precise ratios to create the lignin/PEO mixture.

Nanofiber fabrication was carried out using electrospinning, employing a customizable setup that included a syringe pump, an electrospinning hood, a stainless-steel plate collector, and a high voltage generator (Linari Engineering S.R.L., Pisa, Italy). The process involved the use of a 20 G needle, with

key parameters such as voltage, flow rate, and the distance between the needle and collector carefully adjusted to ensure the production of high-quality fibers (shown in Table 1). Moreover, a fine control of the environmental conditions was adopted, with relative humidity maintained below 9% and a temperature range of 25–28 °C, to facilitate water evaporation and efficient nanofiber formation.

Scanning Electron Microscopy (SEM) Analysis

Electrospun fibers were mounted on stubs with adhesive tape and gold sputter-coated (6 nm gold) with a Leica EM ACE200 gold coater (Leica Microsystems, Wetzlar, Germany) to improve contrast and optimize the sample for imaging. To visualize the fiber, SEM imaging (Hitachi TM3030) was conducted at different magnifications 1000x–7000x, at accelerated voltage of 15 kV. Subsequently, fiber diameters were determined by measuring at least 300 fibers using the open-source ImageJ Fiji software (<https://imagej.net/software/fiji/>) v.1.54, and data were statistically analyzed with the software OriginPro 2024.

Two-Photon Microscopy and FLIM

Two-photon microscopy and FLIM imaging of lignin nanofibers were performed using a Leica TCS SP5 confocal laser scanning microscope equipped with a PicoHarp 300 TCSPC module (Picoquant, Germany). Measurements were acquired using an oil immersion objective (63×/1.4), at a scanning frequency of 400 Hz, and with two-photons excitation wavelength at 840 nm (Spectra-Physics Mai-Tai Ti:Sa ultrafast laser). Confocal images were captured at a resolution of 1024 × 1024 pixels with a detection from 450 nm to 650 nm. Fluorescence emission spectra were recorded from 256 × 256 pixels images, within a detection range of 400–700 nm, using a bandwidth of 10 nm and a step size of 9.38 nm. The microscopy data were processed using the open-source ImageJ Fiji software (<https://imagej.net/software/fiji/>) v.1.54. FLIM analysis was conducted using the phasor method with SimFCS4 software, which was created within the Laboratory of Fluorescence Dynamics, University of California, Irvine (<http://www.lfd.uci.edu>). For FLIM data analysis, the calibration was performed using a sample of fluorescein, which exhibits a fluorescence lifetime of 4 ns (single exponential).

Attenuated Total Reflectance (ATR-FTIR) Spectroscopy and Fourier Transform Infrared Microscopy (micro-FTIR)

Spectra were obtained using a Bruker Lumos ALPHA FTIR spectrometer in Attenuated Total Reflectance mode. Each spectrum was derived from 64 scans, in the range of 4000 to 400 cm⁻¹, with a resolution of 2 cm⁻¹. To ensure that ATR crystal is in close contact with the sample, the nanofiber samples were carefully compressed onto the diamond surface, which cleaned with a 70% ethanol and water solution after each measurement to prevent cross-contamination. Spectra were corrected for

atmospheric compensation through OPUS® software.

Micro-FTIR analysis at room temperature was conducted using a LUMOS Fourier transform infrared microscope (Bruker) with a liquid nitrogen-cooled MCT detector, integrating high-resolution visual imaging via a digital CCD camera. Nanofibers samples were placed between two CaF₂ windows, with a 15 µm Teflon spacer to maintain a consistent path length, and spectra were recorded in transmission mode over a wavenumber range of 4000 to 600 cm⁻¹, averaging 128 scans. The microscope's adjustable aperture was configured to match the regions of interest (ROI), with dimensions of 200 µm × 200 µm.

DPPH Assay for Antioxidant Properties

The antioxidant activity of the samples was assessed through the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay, a commonly used technique that measures the ability of antioxidants to scavenge free radicals. The DPPH radical is a stable free radical distinguished by its intense violet color, due to its unpaired electron, which exhibits strong absorbance at 517 nm. Upon reaction with an antioxidant, the DPPH radical is reduced, leading to a shift in the solution color from violet to pale yellow (Figure 63).

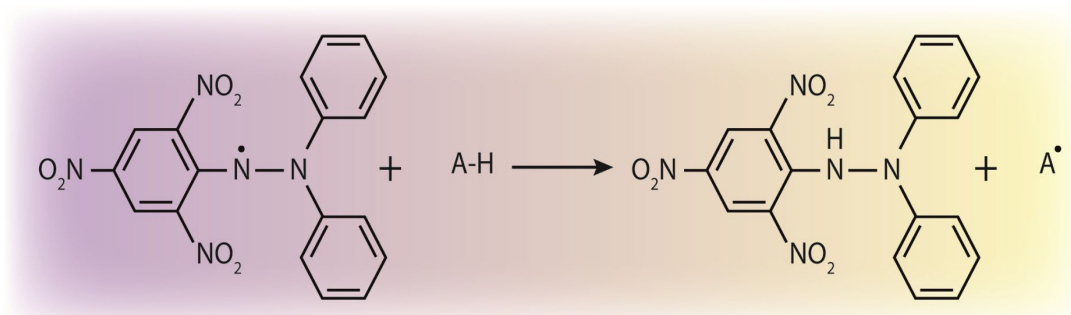


Figure 63: **Mechanism of the DPPH radical scavenging assay** [369,370]. The DPPH radical (DPPH•), characterized by its violet color, reacts with an antioxidant (A-H) that donates a hydrogen atom. This reaction reduces DPPH• to its non-radical form (DPPH-H), resulting in a color change to pale yellow.

The decrease in absorbance at 517 nm is directly related to the scavenging activity of the antioxidant, enabling the quantification of radical scavenging activity (RSA%).

To ensure complete solubilization of the samples, including lignin nanofibers (Lig-NFs), pure lignin (Lig), and ascorbic acid (AA), all were dissolved in a 10:90 water-ethanol mixture prior to testing. This solvent system was selected because pure ethanol alone was insufficient to fully dissolve the nanofibers and lignin, and it also ensured consistency across all sample preparations. Before mixing the samples with the DPPH solution, all were diluted in ethanol to ensure their lignin concentrations fell within the range of 10–2.5 µg/mL, which provides reliable and reproducible results within the linear response range of the assay and preventing absorbance saturation.

A stock solution of DPPH was obtained by dissolving DPPH in ethanol to achieve a concentration of 0.1 mM. For each test, 1 mL of the DPPH solution was combined with 1 mL of the test solution, which

included lignin nanofibers at varying lignin contents (24%, 38%, 48%, and 56%), pure lignin, or ascorbic acid as the positive control. For the nanofibers, the corresponding concentrations of lignin were determined by calculating the dry weight of lignin in the PEO-lignin blend. The DPPH solution mixed with ethanol served as the blank control.

By analyzing preliminary spectra, we observed slight variations in the DPPH spectra in the presence of lignin. For this reason, RSA% was calculated based on the integrated area under the absorbance curve rather than using a single absorbance value at 517 nm. This approach provided a more precise evaluation of antioxidant activity. The RSA% was calculated as follows:

$$\text{Radical Scavenging Activity (\%)} = \frac{(A_{\text{control}} - A_{\text{sample}})}{A_{\text{control}}} \cdot 100$$

where A_{control} represents the integrated area under the curve for the blank control, and A_{sample} represents the integrated area for the test sample.

The mixtures were incubated at room temperature in dark conditions, for specific time intervals (1 h, 24 h, 48 h, and 72 h for the nanofibers; 24 h for pure lignin and ascorbic acid). After incubation, the absorbance spectra were recorded using a UV-Vis spectrophotometer in the range from 300 to 900 nm. Each experiment was conducted in triplicate to ensure reproducibility.

Dynamic Mechanical Analysis (DMA)

Nanofiber mats were tested for mechanical properties using a DMA Q800 (New Castle, DE, USA) in film tension mode. The parameters were selected based on previous studies in literature [104,330]. Rectangular samples (7 × 30 mm) were measured for width and thickness at three points using a digital caliper. A preload force of 0.01 N and an initial displacement of 0.2% were applied to stabilize the samples before testing. The test involved stretching at 0.5 mm/min up to 5 mm. Stress-strain data were analyzed using Thermal Advantage Software v5.5.2 to determine Young's modulus from the linear portion of the stress-strain curve. The experimental setup is shown in Figure 64, where the nanofibers mat is clamped in the DMA Q800 in film tension mode.

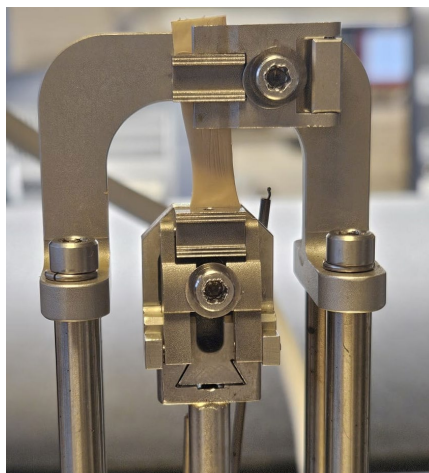


Figure 64: *Experimental setup for mechanical testing of nanofiber mats using the DMA Q800 in film tension mode. The sample is clamped to allow uniaxial tensile testing for the determination of Young's modulus and stress-strain properties.*

Biocompatibility assay

To assess the biocompatibility of lignin-based nanofibers with Human Dermal Fibroblasts (HDF), which were provided from “Istituto Zooprofilattico della Lombardia”, Italy. Cells were grown in DMEM medium which was enriched with 10% FBS, 4 mM of glutamine, and 100 U/mL:100 μ g/mL penicillin:streptomycin.

To evaluate the cell viability Alamar blue (AB) assay was used. HDF were seeded at a density of 8000 cells, in a 96-well plate. About 5 mg discs of Lig/PEO and PEO NFs were dissolved in 150 μ l of DMEM and was added to the cells. After 24 h the AB assay was conducted following the manufacturer's protocol. Briefly, the medium was discarded, the cells were washed with PBS, and 180 μ l of AB solution, prepared in fresh medium, was added to each well. The sample was incubated for three hours, after which the fluorescence of the AB was measured. For the measurement, the parameter was set to an excitation wavelength of 530 nm and emission wavelength of 590 nm, recorded using a microplate reader (Eppendorf AF2200) [371,372].

The data were averaged over 3 replicates per experiment reporting standard deviation and reported as a percentage of the absorbance from the AB solution in the well without any scaffold (control). For each experiment, wells with only the AB solution and no cells were prepared and incubated for 3 hours. The fluorescence readings from these wells were used as the background and subsequently subtracted.

Dissolution Studies

Dissolution studies were conducted by immersing 10 mm diameter discs of Lig-56% nanofibers, prepared using a circular cutter, in water at a concentration of 2 mg/mL. Images for analysis were extracted from a video recorded with a smartphone. The release of lignin from Lig-56% nanofibers was quantified by dissolving the nanofibers in a water volume adjusted to achieve a concentration of 2 mg/mL. At predetermined time points, 20 μ L aliquots were withdrawn and replaced with an equal

volume of ultrapure water to maintain sink conditions throughout the experiment. The withdrawn aliquots were analyzed using a NanoDrop spectrophotometer (ThermoFisher, NanoDrop 2000) ($\epsilon = 22.1 \text{ g}^{-1}\text{L cm}^{-1}$ $\lambda = 280 \text{ nm}$ [368]) (to determine the lignin concentration. The cumulative percentage of lignin released at each time point (t) was calculated as follows:

$$\% \text{ Lignin Released} = \frac{m_{\text{released},t}}{m_{\text{theoretical}}} \cdot 100$$

Where $m_{\text{released},t}$ is the cumulative mass of lignin released at time t , calculated as the sum of the mass of lignin present in the solution at time t and the lignin mass collected from all aliquots withdrawn prior to time t . While $m_{\text{theoretical}}$ is the theoretical total mass of lignin in the nanofiber sample.

Antibacterial Drug Incorporation and Activity in Lignin Nanofibers

Bacitracin was dissolved in ultrapure water, and the same procedure previously described for preparing the lignin/PEO mixture was followed, with the modification of adding the bacitracin solution. The lignin/PEO/bacitracin blend, with a weight-to-weight composition of 51% lignin, 40% PEO, and 9% bacitracin, was stirred for 24 hours prior to electrospinning, which was performed in a cold chamber to prevent bacitracin degradation. The electrospinning parameters required a very low flow rate of 0.4 ml/hour, collector needle distance of 20 cm, and an applied voltage of 17 kV.

Antibacterial assay

The inhibition assay on *Kocuria rhizophila* begins by growing the bacterial strain through streaking on agar plates, which are incubated to allow colony formation. A few well-isolated colonies are then collected using a sterile loop and dispersed in a liquid medium to create a bacterial suspension. The optical density of this suspension is measured at 600 nm to standardize the bacterial concentration. Once the desired OD600 is reached, the suspension is added to LB agar at an appropriate concentration to ensure uniform distribution of the bacteria throughout the medium. The LB agar is poured into sterile Petri dishes and allowed to solidify at room temperature. After the agar has set, the test samples, nanofibers discs, or control solutions, are applied. For liquid samples, 10 μL were pipetted onto the agar surface, while solid samples are gently placed with sterile tweezers. The plates are left to dry until the liquid has completely evaporated. Finally, the Petri dishes are sealed and incubated at 37°C, and the zones of inhibition are observed after 24 and 48 hours.

Chapter 5

Polymeric Electro-sprayed Microparticles for Sustained Release of Ciprofloxacin

This chapter discusses the production and characterization of polymeric microparticles based on Poly(butylene succinate) (PBS) loaded with ciprofloxacin (CPX), prepared by electro-spraying. The release profile of the encapsulated drug was assessed, along with its antibacterial and antibiofilm activities, in particular against skin pathogens. This work was carried out in collaboration with the research group of Professor Mariano Licciardi at the University of Palermo. These results are extensively described in the article from Puleo et al. [373]; here, the aim is to further discuss the same findings presented in the paper to provide additional insights and reflections within the context of this thesis.

5.1 Introduction

Polymeric microparticles (p-MPs) are solid particles which can offer numerous advantages allowing for controlled drug delivery and targeting. Their ability to encapsulate different molecules, such as drugs, active ingredients or other bioactive compounds, have made them particularly valuable. Among the different methods for the preparation of p-MPs, electro-spraying has gained attention as an effective method owing to its simplicity, scalability, and ability to produce particles with fine control over size and morphology. In this chapter, we explore the use of electro-sprayed polymeric microparticles based on Poly(butylene succinate) (PBS), as carriers for the sustained release of ciprofloxacin (CPX), a poor-water soluble antibiotic, commonly used to treat a wide range of bacterial infections. The focus is on the potential of these polymeric microparticles to enhance the therapeutic effectiveness of CPX, improving its solubility and offering a sustained release profile.

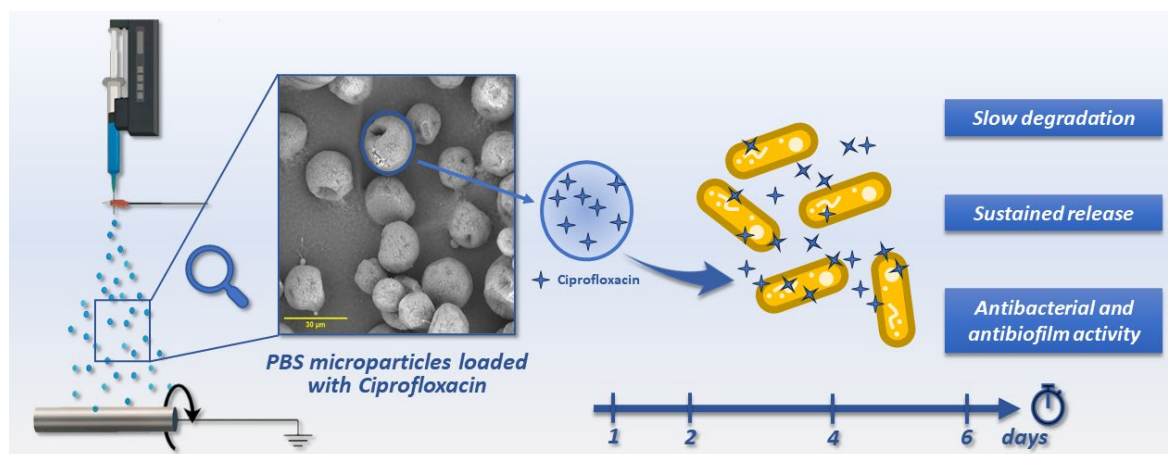


Figure 65: **Graphical representation of Poly-Butyl-Succinate (PBS) microparticles loaded with ciprofloxacin (CPX) for antibacterial applications.** The electro-sprayed microparticles enable sustained drug release, slow degradation rates, and effective antibacterial and antibiofilm activity against pathogens such as *S. aureus* and *P. aeruginosa*. Reprinted with permission from Puleo et al., 2024, under CC-BY license terms [373].

p-MPs are particles based on natural or synthetic polymers which can be produced through different methods, such as solvent evaporation, microfluidics, suspension/emulsion polymerization and electrospray, each offering different advantages [374]. Regardless of the production method, the great potential of PMPs lies in the ability to appropriately select polymers based on the desired features of the final drug delivery system (DDS) [375]. This therefore allows the development of systems with controllable size, shape, morphology, and surface charge, which protect the drug from degradation, and regulate its release under different conditions, such as pH and temperature, but also recognition of specific targets [376–378].

5.1.1 Poly(butylene succinate) (PBS) in Biomedical Applications

Poly(butylene succinate) (PBS) in this study was selected for developing a new drug delivery platform with multiple applications. It is a well-known biodegradable polyester synthesized from renewable resources, which has gained significant attention for its use in biomedical applications [379–382]. Its unique combination of biocompatibility, biodegradability, and easiness of use, positions it as a promising candidate for the production of new biomaterials [380,381].

One of the standout features of PBS is its slow degradation rate, which enables controlled and sustained release of therapeutic agents. This property not only reduces the need for surgical removal of delivery systems but also minimizes environmental impact, making it an environmentally friendly choice.

To this end, different studies report its remarkable potential. For instance, Miceli et al. explored its use in fabricating electrospun tubular scaffolds, which maintained structural integrity over extended periods and supported cell remodelling. These scaffolds exhibited the ability to integrate seamlessly with biological tissues, highlighting PBS's biocompatibility and mechanical robustness [383].

The role of PBS in DSSs has also been extensively studied. Di Prima et al. illustrated its effectiveness by developing microfibrillar scaffolds for ocular inserts. These scaffolds demonstrated controlled release of drugs such as triamcinolone acetonide over a 30-day period, while maintaining critical properties like mucoadhesion and cytocompatibility [384].

From regenerative medicine to advanced DDS, PBS is proving to be a sustainable and adaptable material capable of meeting the demands of modern biomedical challenges.

5.1.2 Ciprofloxacin as Model Drug

Ciprofloxacin (CPX), a broad-spectrum antibiotic belonging to the fluoroquinolone class, is widely used to treat bacterial infections caused by both Gram-positive and Gram-negative pathogens. Its potent antibacterial activity, coupled with its systemic utility, makes it a valuable therapeutic agent in clinical practice [385–387]. Although CPX is not commonly used as a first-line treatment for topical infections, it was specifically chosen as the model drug for this study due to several key factors.

First, ciprofloxacin is a poorly water-soluble drug, which presents challenges in its formulation and delivery [388–390]. This characteristic makes it a suitable candidate for this study, which aims to enhance its solubility and thus bioavailability. Second, while CPX is predominantly employed for systemic administration, its potential for localized delivery remains underexplored. Investigating localized delivery of CPX represents an opportunity to address specific therapeutic needs while potentially reducing systemic side effects and improving localized efficacy [391–393].

In this study, our aim was to evaluate the efficacy of electrospraying as a method for encapsulating CPX as a model antibacterial drug within a polymeric matrix. The encapsulation process was assessed to determine whether the antibacterial properties of CPX were retained post-encapsulation and to characterize the release profile of the drug over time.

5.2 Experimental Results and Discussion

5.2.1 MPs Production and Characterization

The electrospraying protocol was refined during efforts to optimize the production of polybutylene succinate (PBS) structures. During the exploration of different solvent systems for PBS dissolution, conditions were identified that favoured the formation of microparticles rather than nanofibers [373]. To explore this phenomenon, a systematic study was conducted to understand the factors influencing the electrospraying process. As previously described in Chapter 4 for electrospinning, also in this case the key parameters include polymer concentration, solvent characteristics, flow rate, applied voltage, and needle-to-collector distance, were carefully optimized. To this end, the conditions ensuring both high yield and stable operation of the electrospraying process were established. Two distinct types of

PBS-based microparticles were prepared for this study. The first type, referred to as MPs-PBS, consisted of plain PBS microparticles produced without any additional components. The second type, designated as MPs-PBS-CPX, involved PBS microparticles loaded with CPX. These abbreviations will be used throughout the text to differentiate between the empty and CPX-loaded microparticles.

Once the microparticles were produced, their morphology was evaluated using SEM to assess whether CPX encapsulation influenced their structural features. The SEM micrographs in Figure 66 reveal distinct morphological differences between MPs-PBS and MPs-PBS-CPX. The former (Figure 66A) exhibit an irregular and highly porous surface, with some depressions. Conversely, the MPs-PBS-CPX (Figure 66B) display a porous surface morphology with rounded and well-defined shapes, indicative of the structural changes associated with CPX encapsulation. The porosity of these structures represents an interesting feature that may enhance the surface area, which is crucial for improving pharmacokinetic profiles of drugs encapsulated [394,395].

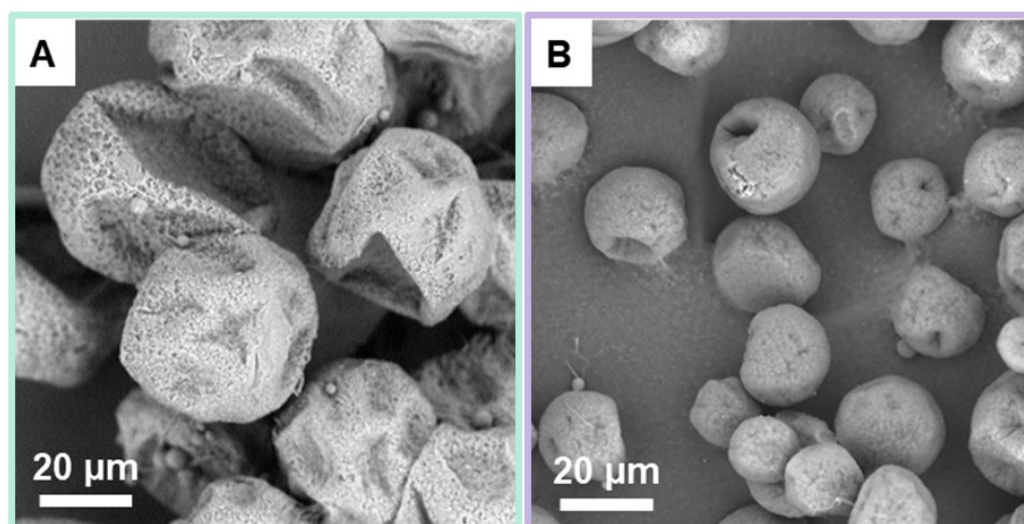


Figure 66: **SEM micrographs** of (A) MPs-PBS microparticles with a porous surface and (B) MPs-PBS-CPX microparticles with a smoother surface. Scale bar: 20 μm . Reprinted and adapted with permission from Puleo et al., 2024, under CC-BY license terms [373].

The particle size distribution is reported in Figure 67 for pristine MPs-PBS a) and MPs-PBS-CPX b). The pristine MPs exhibit an average diameter of $43 \pm 8 \mu\text{m}$, indicating a larger particle size with respect to the MPs-PBS-CPX which instead displays a significantly smaller average diameter of $17 \pm 5 \mu\text{m}$ [373].

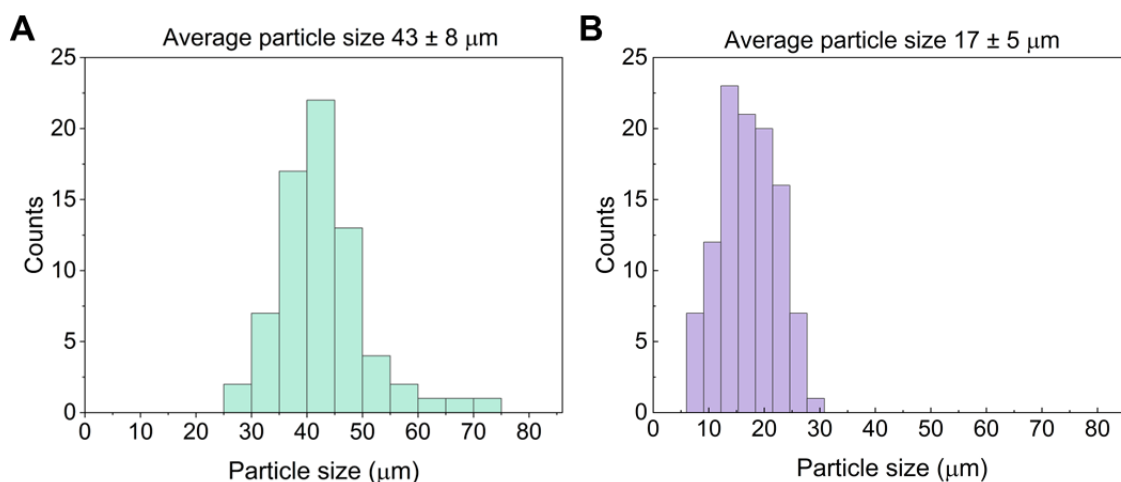


Figure 67: Particle size distribution of microparticles. (A) Loaded microparticles containing CPX exhibit an average particle size of $43 \pm 8 \mu\text{m}$. (B) Empty microparticles have a smaller average particle size of $17 \pm 5 \mu\text{m}$. Readapted with permission from Puleo et al., 2024, under CC-BY license terms [373].

The reduced size of the MPs-PBS-CPX can be explained by differences in the properties of the solution used during electrospraying. The incorporation of CPX may alter the features of the solution such as conductivity, surface tension, or viscosity, which are critical parameters influencing the electrospraying process. For instance, higher conductivity or a lower viscosity or surface tension might result in the formation of smaller droplets during electrospraying, leading to smaller final particle sizes [396,397].

To further investigate the properties of the prepared microparticles, their thermal behavior and chemical composition were characterized using Differential Scanning Calorimetry (DSC) and ATR-FTIR, respectively. In particular, DSC was used to assess the thermal behavior of the microparticles, revealing phase transitions or potential drug-polymer interactions (Figure 68A). The DSC thermograms of MPs-PBS and MPs-PBS-CPX are quite similar, but it is possible to observe for the latter a shift to lower temperatures of the exothermic peak. Such modification can be associated the behaviour of CPX to act as a plasticizer within the polymer matrix, reducing the thermal energy required for molecular mobility [373,398]. These is line with other studies which reported changes in the structural interactions between the polymer chains, disrupting their orderly arrangement, upon drug loading [398,399].

The ATR-FTIR spectra (Figure 68B) further confirmed the successful incorporation of CPX within the microparticles. It was possible to identify peaks attributable to CPX in the MPs-PBS-CPX spectrum, which were absent in MPs-PBS spectrum, such as those at 707 , 822 and 866 cm^{-1} . These peaks are related to the aromatic group of CPX, for instance the C-H aromatic bending vibration [373,400]. In contrast, the empty microparticles (MPs-PBS) exhibited only the typical peaks of the polymer.

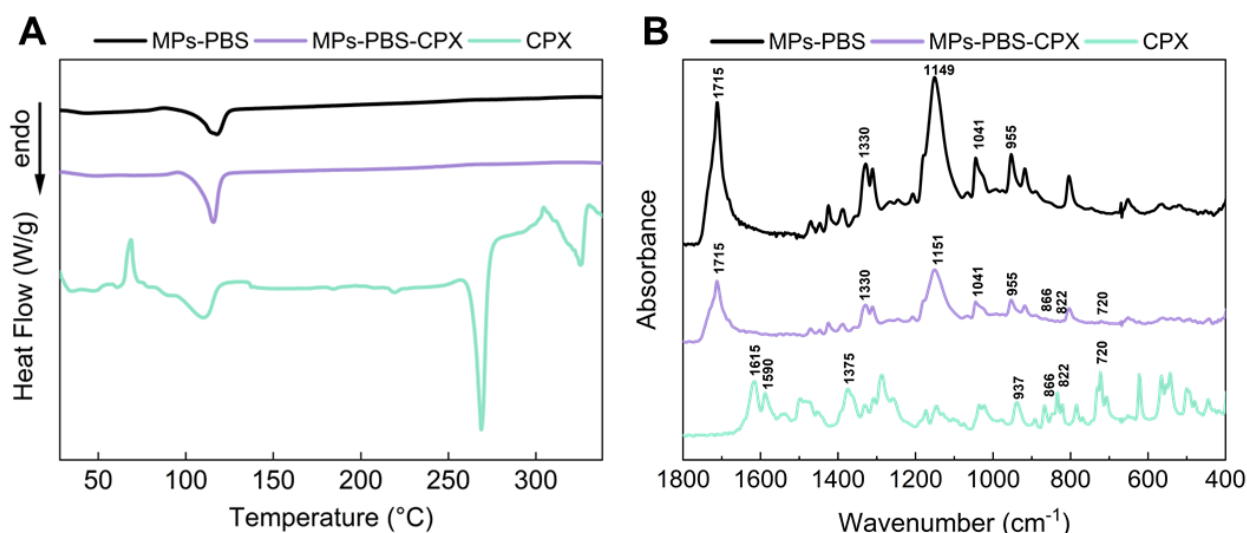


Figure 68: Thermal and chemical characterization of microparticles. (A) DSC thermograms of CPX, MPs-PBS-CPX, and MPs-PBS. The absence of the CPX melting peak in the MPs-PBS-CPX thermogram indicates successful encapsulation. (B) ATR-FTIR spectra of CPX, MPs-PBS-CPX, and MPs-PBS. The characteristic CPX peaks in the MPs-PBS-CPX spectrum confirm its incorporation into the polymeric matrix. Adapted from Puleo et al., 2024, under CC-BY license terms [373].

To comprehensively characterize the solid-state properties of the microparticles in the context of this study and to complement the thermal and chemical analyses, X-ray diffraction (XRD) analysis was performed. This technique is a powerful tool for exploring the structural arrangement of materials at the atomic level, allowing to determine if any crystalline phases persist or if the drug adopts a more disordered, amorphous form (Figure 69). Such characterization is critical in the design of a drug delivery system (DDS), as changes in the solid-state can influence properties such as solubility, dissolution rates, and drug release kinetics [373,400,401].

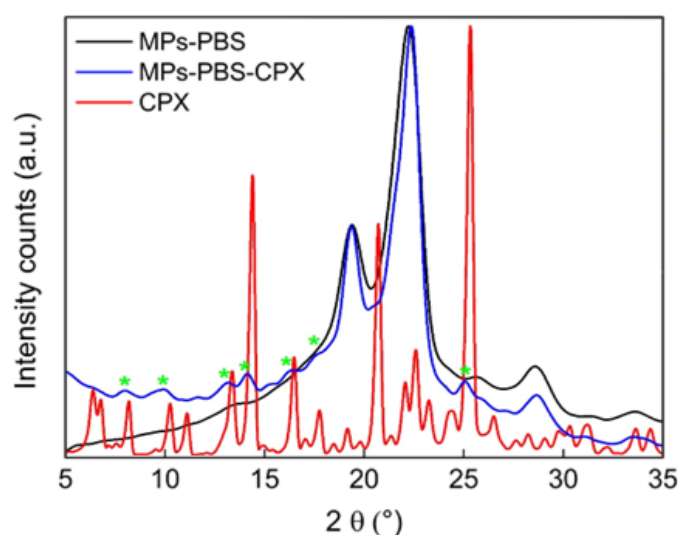


Figure 69: XRD patterns of MPs-PBS (black), MPs-PBS-CPX (blue), and CPX (red) are shown. Characteristic diffraction peaks of CPX are indicated by green stars, confirming its presence within the MPs-PBS-CPX sample. Reprinted with permission from Puleo et al., 2024, under CC-BY license terms [373].

The XRD patterns revealed characteristic peaks of CPX, confirming its presence in the loaded MPs. However, these peaks showed reduced intensity, likely because the CPX concentration within the MPs

was relatively low. Additionally, to further investigate the effects in the solid-state organization induced by CPX incorporation the degree of crystallinity (DC) was calculated. This parameter represents the proportion of crystalline regions within the total material, significantly impacting the physical and functional properties of the MPs [400,401]. As reported in Puleo et al. [373], a comparison of DC values revealed a decrease from 59.2% in empty MPs to 39.6% in CPX-loaded MPs, while pure CPX exhibited a DC of 60.2%. This reduction in crystallinity indicates that CPX incorporation disrupts the ordered arrangement of the PBS matrix, leading to a more amorphous structure. Overall, the morphological and solid-state characterizations collectively demonstrate that CPX encapsulation alters the properties of the PBS polymer matrix within the microparticles [400,402].

While these analyses provide a fundamental understanding of the material's properties, it is also essential to assess how these structural characteristics translate into stability and behaviour in biological environments. To this end, the stability of the microparticles (MPs-PBS) was evaluated through a degradation study conducted in phosphate-buffered saline (DPBS) and plasma over a 30-day period (Figure 70), considering that this material may be used for prolonged treatments.

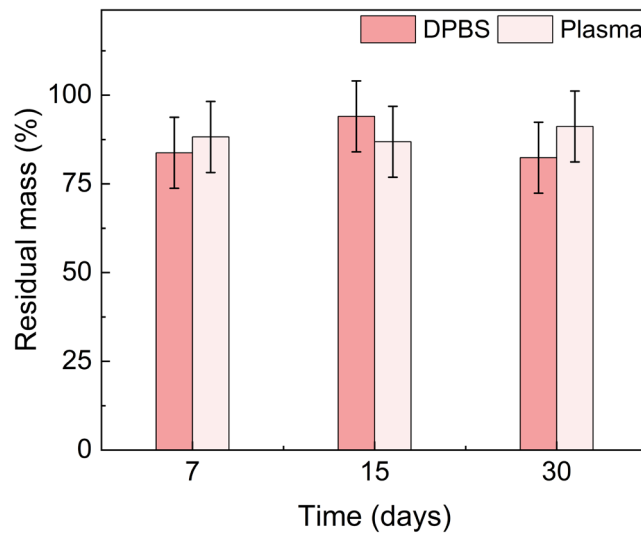


Figure 70: **Degradation study of microparticles in DPBS and plasma.** Residual mass (%) of microparticles MPs-PBS, measured after a period of incubation of 7, 15, and 30 days. The microparticles exhibit a slow degradation profile, with over 75% residual mass maintained in both media, demonstrating a slow and stable degradation profile. Adapted from Puleo et al., 2024, under CC-BY license terms [373].

The microparticles retained over 75% of their residual mass throughout the 30-day study period in both DPBS and human plasma, highlighting their slow and controlled degradation profile. The use of DPBS provides a simplified medium that mimics physiological ionic strength and pH, while human plasma represents a more complex biological environment, closer to in vivo conditions [382,383]. This dual approach ensures a comprehensive evaluation of the microparticles' stability under conditions relevant to potential biomedical applications. This stability is a promising feature, as it suggests a

reduced risk of triggering an inflammatory response and supports their potential suitability for applications in tissue regeneration, as outlined from other studies regarding PBS [380,383].

5.2.2 Investigation of Dissolution and Permeation of CPX from MPs-PBS-CPX

The release profile of CPX from the microparticles was evaluated through a biphasic dissolution study conducted in a water-octanol system, as illustrated in the methods section (Figure 74). This approach allows for the simulation of drug partitioning between hydrophilic (water) and lipophilic (octanol) environments, offering valuable information on release kinetics. The aqueous phase represents bodily fluids, while the octanol phase serves as a model for lipophilic environments such as cell membranes or tissues [373,403]. Figure 71A illustrates the results of this study, demonstrating the release of CPX from the microparticles over time compared with the free CPX.

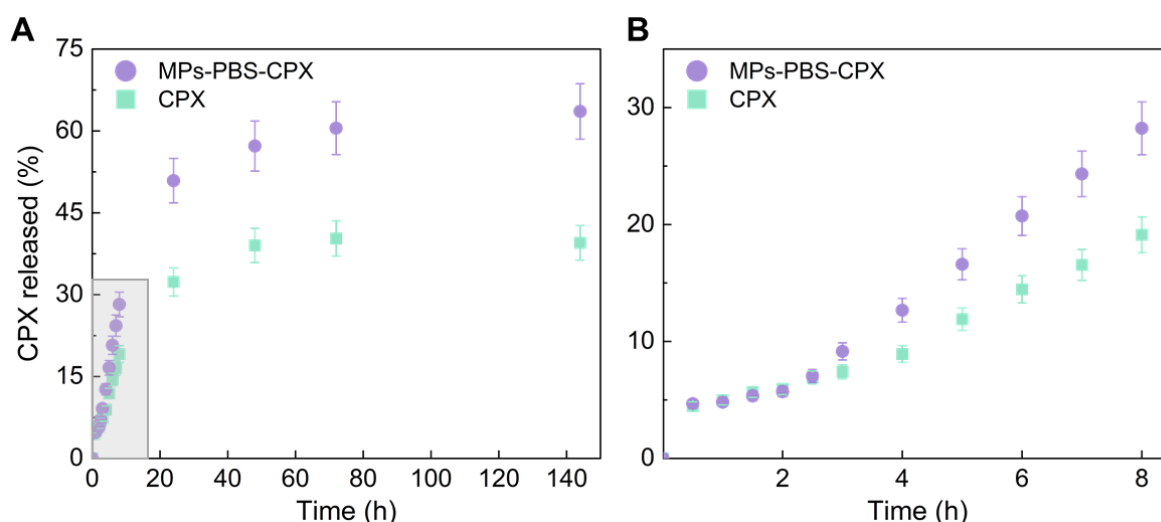


Figure 71: Drug release profiles of CPX in a biphasic water/octanol system over time. (A) Cumulative release of CPX over 140 hours shows a sustained and controlled release from microparticles (MPs-PBS-CPX) compared to free CPX. (B) Early release phase (first 8 hours) demonstrates the gradual release of CPX from microparticles, in contrast to the more rapid release of free CPX. The presence of CPX in the octanol phase indicates successful partitioning into the lipophilic environment. Adapted from Puleo et al., 2024, under CC-BY license terms [373].

The release profiles demonstrate that CPX encapsulated in microparticles (MPs-PBS-CPX) exhibited a sustained release compared to free CPX. A closer examination of the early release phase (Figure 71B) highlights that the microparticles gradually release CPX during the first eight hours, compared to the more rapid release observed for free CPX. This controlled release can be attributed to the different crystalline states in which the CPX is present within the polymer matrix, which leads to a different dissolution profile [373]. This observation is consistent with other CPX delivery systems, where changes in the drug's solid-state form have been shown to influence dissolution profiles significantly [404,405]. Moreover, as the PBS matrix gradually disintegrates, it further supports the prolonged release of the drug, making this system an effective sustained drug delivery method. It can be hypothesized that the release mechanism primarily occurs through diffusion, highlighting the ability

of the microparticles to regulate CPX release over extended periods [373,389,402].

Additionally, the presence of CPX in the lipophilic octanol phase confirms the ability of the microparticles to facilitate the transfer of CPX to hydrophobic environments, further validating their potential for biomedical applications.

With the aim of assessing how the drug permeates from MPs-PBS-CPX through biological barriers, permeation experiments were carried out using an ex-vivo porcine skin model in a Franz diffusion cell, compared to the free CPX. This study serves to further validate the dosing system for topical applications such as skin infections or for transdermal delivery [406,407]. The setup, presented in the methods section in Figure 75 mimics the barrier properties of human skin and provides valuable information on the possibility of using these microparticles as drug delivery platform [403,408,409]. The total quantity of CPX that effectively permeated the skin over time, along with the distribution of CPX across the different compartments (donor and acceptor solution, and the skin membrane) were analyzed (Figure 72).

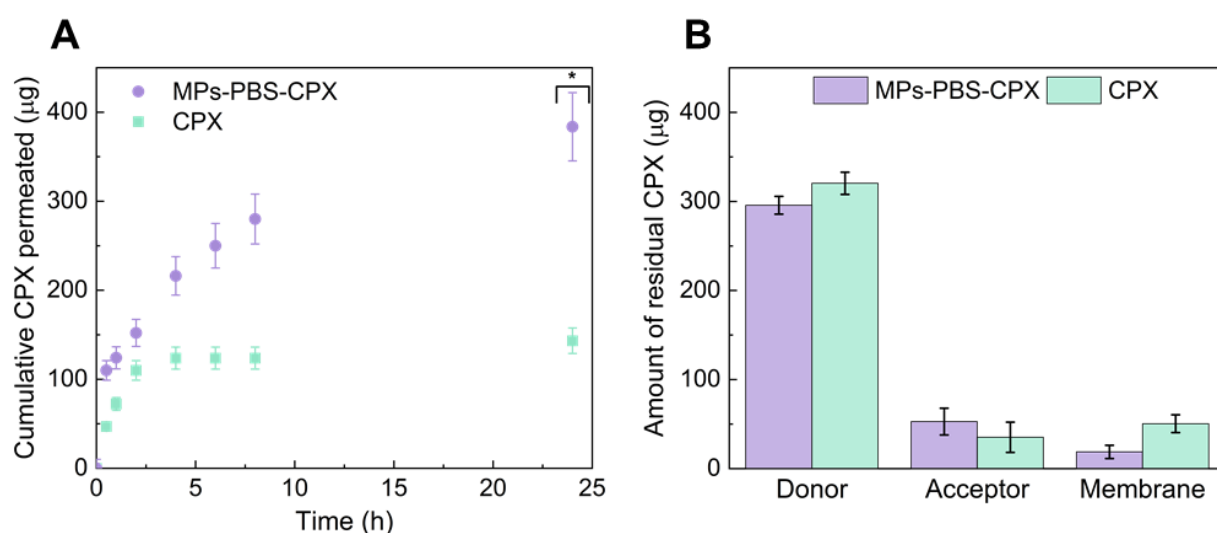


Figure 72: Permeation of CPX through porcine skin using Franz diffusion cells. (A) Cumulative CPX permeated over 24 hours shows significantly higher permeation for MPs-PBS-CPX compared to free CPX (* $p < 0.05$). (B) Distribution of residual CPX in the different compartment of the assay: in the donor and acceptor solutions and in the solution extracted from the skin membrane. MPs-PBS-CPX exhibit enhanced permeation into the acceptor solution, while free CPX shows a higher residual concentration in the donor solution. Adapted from Puleo et al., 2024, under CC-BY license terms [373].

The cumulative permeation profile (Figure 72A) shows an enhanced permeation of CPX over 24 hours through the skin when encapsulated in microparticles (MPs-PBS-CPX). This observation is in contrast with what was observed for the free drug, which shows slower permeation, potentially leading to drug accumulation and precipitation within the skin membrane, limiting its effective distribution [373]. To this end, ensuring a good permeation profile of the drug is crucial for avoiding accumulation and supporting a gradual and extended-release through the skin, a favorable feature for transdermal drug delivery systems.

To better evaluate how the drug distributes among the different compartments of the assay, a comparison of the residual CPX levels between donor, acceptor, and membrane was performed, revealing key differences (Figure 72B). The microparticles result in lower drug levels remaining in the donor compartment, when in comparison with the CPX alone, due to their efficient release mechanism [373]. This phenomenon, for the sample of MPs-PBS-CPX results in an increased concentration of CPX in the acceptor compartment, meaning that more drug was able to permeate and thus confirming the ability of the microparticles to facilitate controlled and extended transdermal delivery.

5.2.3 Investigation of Antibacterial and Antibiofilm Properties of MPs-PBS-CPX

The evaluation of antibacterial activity is crucial for determining the efficacy of potential antimicrobial DDS. In this context, the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) against several bacterial strains using different growth media were tested *in vitro*. This approach, which included Mueller-Hinton (MH), Mueller-Hinton II (MHII), and Tryptic Soy Broth (TSB), the study aims to account for variations in bacterial growth conditions, ensuring a comprehensive assessment of CPX efficacy against different bacterial strains under diverse environmental scenarios [410,411]. The results, summarized in Table 3 providing detailed data on the antibacterial performance of CPX under varying conditions.

Strains	MIC ($\mu\text{g/mL}$)		
	MH medium	MHII medium	TSB medium
<i>S. aureus</i> ATCC 25923	12.5	1.5	6.2
<i>P. aeruginosa</i> ATCC15442	12.5	0.75	12.5
<i>S. hominis</i> ATCC 27844	1.5	1.5	3.1
<i>S. epidermidis</i> ATCC 12228	6.2	6.2	3.1
<i>C. acnes</i> ATCC 11827	12.5	12.5	12.5
<i>S. agalactiae</i> ATCC 13813	3.1	1.5	3.1

Table 3: **Values of MIC ($\mu\text{g/mL}$)** of MPs-PBS-CPX evaluated through an *in vitro* assay in which different culture media growth (MH, MHII, TSB) were used, and the effects against different bacterial strains were observed. In particular, *S. hominis*, *S. epidermis*, *C. acnes* and *S. agalactiae* were selected as strains associated with skin infection. Reprinted from Puleo et al., 2024, under CC-BY license terms [373].

The MIC values indicate that the antibacterial activity of ciprofloxacin-loaded microparticles was more pronounced in the Mueller-Hinton II (MHII) medium against *S. aureus* and *P. aeruginosa*. In TSB, the MIC values for these strains were comparable to those observed in Mueller-Hinton (MH) medium. This is interesting as it suggests that, despite TSB being richer in nutrients and therefore providing a greater opportunity for bacterial strain proliferation, it does not alter the antibacterial efficacy of the

delivery system [373]. For dermatological strains, including *Staphylococcus hominis*, *Staphylococcus epidermidis*, *Cutibacterium acnes*, and *Streptococcus agalactiae*, no significant differences in susceptibility were observed across the tested media. These strains were chosen because they are commonly associated with skin-related infections, making them highly relevant for assessing the potential of the microparticles in skin-infections related applications [412,413].

To complement the MIC studies, the Minimum Bactericidal Concentration (MBC) of CPX was also determined for the same bacterial strains and growth media (MH, MHII, and TSB) to assess its bactericidal efficacy. The MBC values are presented in Table 4.

Strains	MBC ($\mu\text{g/mL}$)		
	MH medium	MHII medium	TSB medium
<i>S. aureus</i> ATCC 25923	50	50	>100
<i>P. aeruginosa</i> ATCC15442	12.5	3.1	25
<i>S. hominis</i> ATCC 27844	50	>100	100
<i>S. epidermidis</i> ATCC 12228	50	12.5	50
<i>C. acnes</i> ATCC 11827	100	50	100
<i>S. agalactiae</i> ATCC 13813	50	25	50

Table 4: **MBC ($\mu\text{g/ml}$) values** measured *in vitro*, derived from MICs assessed across various growth media (MH, MHII, TSB) for the different bacterial strains tested. Reprinted from Puleo et al., 2024, under CC-BY license terms [373].

Improved antibacterial activity was observed in the MHII medium for both *S. aureus* and *P. aeruginosa*, indicating a favourable environment for the assay. In the case of the Gram-positive strain *P. aeruginosa*, the MIC and MBC values were identical when tested in MH growth medium, suggesting consistent pharmacological efficacy under these conditions. A different observation can be made with TSB medium, which influenced the MBC values against *S. aureus* and skin-related strains, requiring higher concentrations ($\mu\text{g/mL}$) to effectively eradicate the strains [373]. However, these concentrations represent a good outcome compared to the doses usually required for CPX and are consistent with what has been reported in the literature [387,414–416]. These encouraging results, combined with the improved drug release profile, suggest that microparticles may represent a valid dosing system with potential for further exploration in *in vivo* studies, such as models simulating infected wounds or chronic infections.

Lastly, the ability of the microparticles to inhibit *P. aeruginosa* biofilm formation was further evaluated by testing their activity at sub-inhibitory concentrations (sub-MIC). The results showed that, the MPs-PBS-CPX achieved a significant biofilm inhibition rate of 52.5% [373]. as the main challenge in clinically treating *P. aeruginosa* infections is the formation of biofilms, which make these infections

persistent and, most importantly, resistant to treatment.. The observed inhibition rate aligns closely with values reported in similar studies, reinforcing the efficacy of MPs-PBS-CPX as a promising strategy for managing biofilms [417,418].

5.3 Conclusions

The polymeric microparticles developed in this study have demonstrated exceptional efficacy as a drug delivery system, effectively encapsulating and releasing ciprofloxacin (CPX), a poorly water-soluble antibiotic. By leveraging the electrospraying technique, PBS-based microparticles were successfully produced, exhibiting good properties of drug encapsulation, sustained release, and a notable decrease in CPX crystallinity. The slow degradation rate of the PBS polymer enhances biocompatibility and ensures prolonged drug release, making these microparticles potentially suitable for applications in wound healing and chronic infection management.

The system's ability to counteract biofilm formation by *P. aeruginosa* and improve skin permeation of CPX, as evidenced by permeation studies on porcine skin, further underscores its potential as an advanced platform for topical antibacterial therapies. Additionally, the observed antibacterial efficacy against skin-related bacteria, which often contribute to complications in conditions like diabetic foot ulcers and unhealed surgical wounds, highlights the versatility and robustness of this drug delivery system.

To further explore this system in antibacterial therapy, future research could explore the incorporation of photosensitizing or photocatalytic agents into the microparticles for applications in photodynamic therapy. The system's excellent drug-loading capabilities and sustained delivery properties make it an ideal candidate for combining traditional and light-activated therapeutic approaches, potentially broadening its applicability to other multidrug-resistant infections.

In conclusion, these findings position PBS-based microparticles as a highly effective drug delivery model with significant potential for further development. Future studies should also investigate in vivo performances and explore the co-loading of multiple therapeutic agents.

5.4 Experimental Methodology

For a detailed description of the experimental methodologies, refer to article [373]. However, the main methods are briefly outlined here.

Materials

Poly(1,4-butylene succinate) (PBS), extended with 1,6-diisocyanatohexane, ciprofloxacin (CPX), n-octanol, Dulbecco's phosphate buffered saline (DPBS), Mueller–Hinton broth (MHB), Mueller–Hinton broth (MHBII), tryptic soy agar (TSA), and crystal violet were sourced from Sigma,

Italy. Chloroform was obtained from VWR Chemicals. For in vitro antibacterial tests, the following strains were used: *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 15442 (official reference strains for UNI EN European Standards), and skin-associated bacteria including *Staphylococcus hominis* ATCC 27844, *Staphylococcus epidermidis* ATCC 12228, *Cutibacterium acnes* ATCC 11827, and *Streptococcus agalactiae* ATCC 13813, all purchased from PBI-VWR.

Electrospraying for Microparticles Production

Briefly, microparticles were prepared by dissolving PBS (15% w/v) in chloroform, alone or with the addition of ciprofloxacin (CPX, 5% w/w), through vortexing and sonication. The resulting solution was electrosprayed using an NF-103 electrospinning apparatus (MECC, Japan) at room temperature. The process parameters, including electric field, feed rate, and collector setup, were optimized and are detailed in [373]. After production, microparticles were detached from the collector, and kept at ambient temperature for subsequent examination. The electrospraying instrumental setup is illustrated in Figure 73.

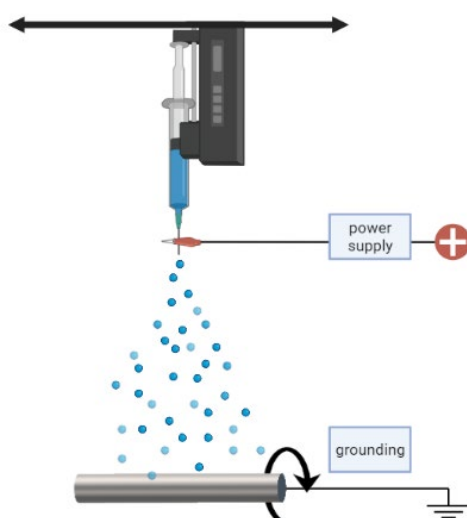


Figure 73: Schematic representation of the electrospraying process used for microparticle preparation. Created with Biorender.com. Reprinted from Puleo et al., 2024, under CC-BY license terms [373].

A polymer-drug solution is extruded through a syringe attached to a flat-tip needle under an applied voltage (kV) from a power supply. The charged droplets are directed towards a grounded rotating collector, where microparticles are deposited..

Microparticles Characterization

The morphology of the microparticles was assessed using SEM imaging (Phenom ProXSEM), equipped with EDX. Samples were prepared on adhesive tape, dried under vacuum, and analyzed at 10 kV. The average particle diameter was calculated from at least 300 measurements from different images using ImageJ software.

X-ray Powder Diffraction (XRD)

XRD patterns were measured using a Bruker D8 Advance diffractometer, with a Cu K α radiation source ($\lambda = 1.54 \text{ \AA}$), covering a 2θ range of 5° – 35° . The degree of crystallinity was analyzed using Match! 3 software.

Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy (ATR-FTIR)

ATR-FTIR spectra were obtained with a Bruker Lumos FTIR spectrometer. Measurements were acquired in the range of 4000 – 400 cm^{-1} at 2 cm^{-1} resolution. Baseline correctionsfigure 28

were applied using OPUS[®] software.

Differential Scanning Calorimetry (DSC)

Thermal properties were evaluated using a Setaram DSC131 EVO, with heating rates of $20 \text{ }^\circ\text{C/min}$ over 20 – $300 \text{ }^\circ\text{C}$, analyzing MPs with and without CPX, as well as pure CPX.

Degradation Studies

Microparticle degradation was tested at $37 \text{ }^\circ\text{C}$ and $5\% \text{ CO}_2$ in DPBS containing $0.05 \text{ \% w/v}$ sodium azide and plasma. Samples ($\sim 20 \text{ mg}$) were incubated in 1 mL of medium and analyzed at 7, 15, and 30 days. After incubation, samples were washed, filtered, freeze-dried, and weighed to calculate the residual mass. Results were averaged over three replicates and plotted against the initial time point.

In Vitro Dissolution Studies

The release of ciprofloxacin from the microparticles was evaluated using a biphasic medium composed of 100 mL of DPBS to which were added $0.05\% \text{ w/w}$ Tween and 25 mL of *n*-octanol. Subsequently, a fixed amount of MPs and a correspondent amount of CPX were added in a stainless steel 40-mesh metal basket within a beaker, as illustrated in Figure 74. The system was stirred and maintained at $37 \text{ }^\circ\text{C}$ for six days. Samples of the organic phase (*n*-octanol) were collected at different time points, analyzed via UV-Vis spectrophotometry at 282 nm , and returned to the system to maintain equilibrium.

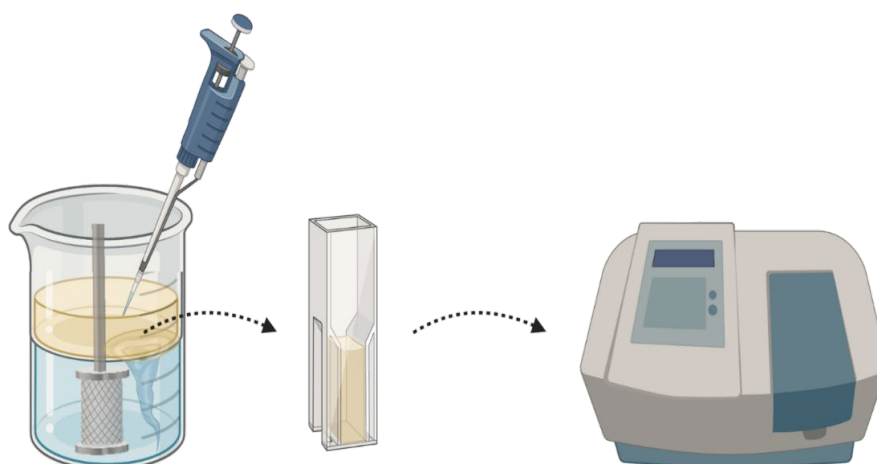


Figure 74: **Experimental setup for in vitro dissolution studies.** The biphasic system includes a stainless-steel mesh basket containing the microparticles or the free CPX, immersed in a mixture of DPBS and n-octanol within a beaker, maintained under stirring at 37 °C. Samples from the organic phase (n-octanol) are collected at different time points using a pipette, transferred to a cuvette, and analyzed by UV-Vis spectrophotometry to determine CPX concentrations at 282 nm. Created with Biorender.com. Reprinted from Puleo et al., 2024, under CC-BY license terms [373].

Assessment of Drug Permeation Through Porcine Skin

The permeation profiles were evaluated through an ex-vivo protocol, using vertical Franz diffusion cells with porcine skin as a model for transdermal drug delivery. Figure 75 illustrates the assembly of the Franz cell with porcine skin between the donor and acceptor compartments used for this assay. The porcine skin morphology was analyzed using a Dino-Lite digital microscope, confirming its integrity throughout the experiment.

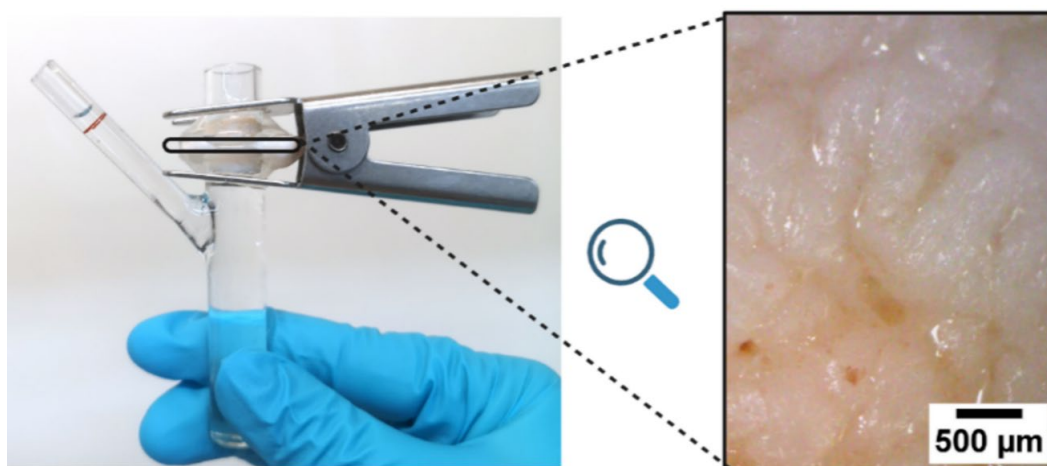


Figure 75: **Vertical Franz diffusion cells with porcine skin in between for ex-vivo experiment to evaluate drug permeation profiles** The magnified image shows the surface morphology of the porcine skin after the study, with a scale bar of 500 μm . Reprinted from Puleo et al., 2024, under CC-BY license terms [373].

MIC and MBC Evaluation

The minimum inhibitory concentrations (MICs) of the microparticles were determined using the broth microdilution method against six bacterial strains (*S. aureus*, *P. aeruginosa*, *S. hominis*, *S. epidermidis*, *C. acnes*, and *S. agalactiae*). Serial dilutions were prepared in three different media (Mueller Hinton

Broth, Mueller Hinton Broth II, and Tryptic Soy Broth) within a 96-well plate. After inoculating bacterial suspensions and incubating for 24 hours at 37 °C, the optical density (OD) was measured at 570 nm to determine the MIC.

The minimum bactericidal concentrations (MBCs) were established by sub-culturing samples from MIC negative wells onto TS agar plates, which were subsequently incubated for 24 hours at controlled temperature of 37 °C. The lowest concentration yielding minimal bacterial growth (≤ 3 colonies) was considered the MBC.

Biofilm Susceptibility Testing

The assay to evaluate whether the microparticles produced here are capable of inhibiting biofilm formation was determined using sub-MIC concentrations (1 to 0.05 $\mu\text{g/mL}$) of *P. aeruginosa*. Biofilms were formed in a glucose-enriched TSB medium in 96-well plates. After formation the biofilms were stained with crystal violet which allowed for quantification of the biofilm biomass after incubation for 24 hours at 37 °C. The results of the assay were recorded spectrophotometrically at 600 nm.

Chapter 6

Conclusions and Future Perspectives

In this thesis the various facets of innovative nano and micro materials have been explored by studying three distinct systems: nanostructured composites based on nitrogen-doped titanium dioxide (N-TiO₂) and gold nanorods (AuNRs), sustainable lignin-based nanofibers and polymeric microparticles loaded with ciprofloxacin. These studies integrate innovative material design with biophysical characterization and functional evaluation, laying a robust foundation for the development of multifunctional systems that address critical challenges in antibacterial therapy and biomedical applications.

The research into N-TiO₂ and its composite with anisotropic AuNRs has demonstrated the synergistic potential of combining materials to enhance performance (**Chapter 3**). N-TiO₂, characterized by its heterogeneous morphology and micrometric aggregates, provides a robust platform for photocatalytic activity, while the inclusion of AuNRs, synthesized for their unique plasmonic properties and anisotropic shape, significantly enhances the composite's efficiency. The physical mixing of these two components, facilitated by their opposing surface charges, offers a straightforward yet effective approach to material integration. This composite system demonstrated superior photocatalytic activity compared to N-TiO₂ alone, as evidenced by its enhanced ability to degrade methyl orange, a model organic dye. The improved performance can be attributed to the stabilization of photogenerated electron-hole pairs, reducing recombination and increasing the efficiency of the oxidative processes. Our approach aimed at further investigating N-TiO₂/AuNRs composite capabilities of oxidation, particularly related to the main bacterial components. The ability to oxidize DNA molecules was confirmed through spectroscopic analysis, which revealed significant structural alterations, including the unfolding of the DNA double helix and the oxidation of nucleotide bases. These findings were coupled with studies in lipid membrane oxidation, using fluorescence-based techniques with model vesicles. These investigations showed faster oxidation rates and significant changes in membrane properties, such as increased rigidity and dehydration. To translate these findings into practical applications, a chitosan-based hydrogel embedded with the photocatalytic materials was fabricated. This hybrid system retained the composite's robust oxidative properties, demonstrating its potential for use in biomedical contexts, such as wound dressings or implant coatings.

Parallel to these advancements, the study of lignin-based nanofibers focused on the sustainable development of functional biomaterials (**Chapter 4**). Lignin, a natural byproduct with significant antioxidant properties, often discarded due to its complex processability, was successfully valorized through a green fabrication approach that employed water as the sole solvent. The methodology relied on pH adjustments and ultrasonication to improve lignin's dispersibility, followed by blending with PEO to enable electrospinning. This approach yielded nanofibers with varying lignin content, achieving concentrations of up to 56 wt%. Structural analysis revealed the influence of lignin concentration on fiber morphology and diameter, demonstrating the system's versatility and adaptability for different applications.

The lignin-based nanofibers exhibited a range of valuable properties, including intrinsic autofluorescence, which enabled advanced imaging through two-photon confocal microscopy and fluorescence lifetime imaging. These techniques provided insights into the internal structural organization of the fibers, differentiating between densely packed and less concentrated lignin regions. Infrared spectroscopy revealed strong interactions between lignin and PEO, supporting fiber stability and formation. Importantly, the fibers demonstrated sustained release of lignin over time, aligning with its antioxidant activity, which was retained for up to three days. Mechanical characterization showed that higher lignin content reduced fiber rigidity, offering a tuneable balance between flexibility and stiffness, which is critical for tailoring materials to specific biomedical needs. The biocompatibility of these nanofibers was confirmed through human fibroblast assays, and their ability to encapsulate and release bacitracin, a model antibacterial drug, while maintaining its efficacy, underscores their potential as controlled drug delivery systems.

Furthermore, polymeric microparticles produced through electrospraying demonstrated their potential as a platform for sustained drug delivery (**Chapter 5**). This method offers promising opportunities for therapeutic applications by enabling the gradual and precise release of drugs according to specific requirements. The microparticles were successfully produced, showing good drug-loading efficiency. A detailed solid-state characterization confirmed that the drug was effectively incorporated into the polymeric matrix, ensuring a stable and homogeneous distribution. Additionally, changes in the crystalline-to-amorphous state of the drug were observed, which influenced its solubility properties, which is a critical aspect for enhancing the bioavailability of the drug. Ciprofloxacin was used as a model drug in this study, demonstrating the effectiveness of the system. However, this approach could also be adapted for other therapeutic agents or for combinations of drugs, providing a flexible platform for diverse applications.

Given these promising results, it is worth investigating in different directions for further development of multifunctional antibacterial materials. In the case of N-TiO₂/AuNRs composites, further research could explore the functionalization of N-TiO₂ with gold, in its metallic form or as pre-formed NPs, directly during the synthesis to further optimize the composite. Another approach could also consider functionalizing the surfaces opportunistically to create covalent linkages, which could enhance the stability and efficacy of these composites. Furthermore, comparative studies in more complex lipid membranes with varied compositions could further elucidate their performance in biologically relevant environments, coupled with evaluation on bacterial cultures. Ultimately, these systems could be tested on chronically infected wounds that are difficult to heal to evaluate their antibacterial efficacy and to promote tissue healing by eradicating the infection.

Similarly, future work on lignin-based nanofibers could focus on developing multilayer electrospun systems that integrate the antioxidant properties of lignin with polymeric layers offering enhanced water resistance. These multilayer systems could find applications in wound healing, where multifunctionality is essential. Alternatively, another area of research to explore could involve loading different drugs, such as other poorly soluble antibiotics like rifampicin and mupirocin, as these medications are often challenging to deliver effectively due to their limited solubility, and improving their bioavailability could enhance their therapeutic outcomes in treating infections [419,420]. These molecules would benefit from advanced delivery systems to overcome challenges related to solubility, stability, and targeted release, ultimately enhancing their therapeutic potential [421–423].

For polymeric microparticles, it would be equally beneficial to investigate the encapsulation of the same molecules mentioned above, including both antibiotics and anti-inflammatory agents, in order to evaluate their delivery performance within this dosing system. Furthermore, this approach would enable a comparative analysis of the release profiles between microparticles and nanofibers. Additionally, studying the interaction of these microparticles with biological environments would be a critical step, including conducting *in vivo* evaluations or simulating wound conditions to better understand their behaviour, efficacy, and safety under realistic scenarios.

Another significant direction for future research involves optimizing this platform to integrate with smart stimulus-responsive strategies. For example, systems that release drugs in response to specific triggers (e.g., pH, temperature, or enzymatic activity) or designing targeted delivery mechanisms to direct drugs precisely to the affected area could further enhance the therapeutic potential of these microparticles. These approaches would not only improve the efficacy of the drug delivery system but also broaden its applicability in precision medicine and wound care.

In conclusion, this thesis demonstrates how a multidisciplinary approach that integrates biophysics, chemistry, and pharmaceutical sciences in the design, preparation, and characterization of new antibacterial therapeutic systems is essential for understanding the specific mechanisms required for effective antibacterial activity. The combination of these disciplines has allowed for a comprehensive investigation of both the physicochemical properties and the biological interactions that govern the efficacy of antibacterial agents.

Through the application of advanced biophysical techniques, this work has elucidated key structural and dynamic features that influence the interaction between therapeutic systems and bacterial targets. The chemical strategies employed have enabled the synthesis of innovative compounds with improved stability, selectivity, and activity profiles. Furthermore, the pharmaceutical perspective has provided critical insights into formulation optimization and the potential translational applications of these systems.

Overall, the findings presented in this thesis highlight the importance of an integrated scientific approach to tackle the growing challenge of antibiotic resistance.

Bibliography

- [1] S. Mudenda, B. Chabalenge, V. Daka, R.L. Mfuné, K.I. Salachi, S. Mohamed, W. Mufwambi, M. Kasanga, S.K. Matafwali, Global Strategies to Combat Antimicrobial Resistance: A One Health Perspective, *Pharmacology & Pharmacy* 14 (2023). <https://doi.org/10.4236/pp.2023.148020>.
- [2] M. de Mesquita Souza Saraiva, K. Lim, D.F.M. do Monte, P.E.N. Givisiez, L.B.R. Alves, O.C. de Freitas Neto, S. Kariuki, A.B. Júnior, C.J.B. de Oliveira, W.A. Gebreyes, Antimicrobial resistance in the globalized food chain: a One Health perspective applied to the poultry industry, *Brazilian Journal of Microbiology* 53 (2022). <https://doi.org/10.1007/s42770-021-00635-8>.
- [3] C.J. Murray, K.S. Ikuta, F. Sharara, L. Swetschinski, G. Robles Aguilar, A. Gray, C. Han, C. Bisignano, P. Rao, E. Wool, S.C. Johnson, A.J. Browne, M.G. Chipeta, F. Fell, S. Hackett, G. Haines-Woodhouse, B.H. Kashef Hamadani, E.A.P. Kumaran, B. McManigal, R. Agarwal, S. Akech, S. Albertson, J. Amuasi, J. Andrews, A. Aravkin, E. Ashley, F. Bailey, S. Baker, B. Basnyat, A. Bekker, R. Bender, A. Bethou, J. Bielicki, S. Boonkasidecha, J. Bukosia, C. Carvalheiro, C. Castañeda-Orjuela, V. Chansamouth, S. Chaurasia, S. Chiurchiù, F. Chowdhury, A.J. Cook, B. Cooper, T.R. Cressey, E. Criollo-Mora, M. Cunningham, S. Darboe, N.P.J. Day, M. De Luca, K. Dokova, A. Dramowski, S.J. Dunachie, T. Eckmanns, D. Eibach, A. Emami, N. Feasey, N. Fisher-Pearson, K. Forrest, D. Garrett, P. Gastmeier, A.Z. Giref, R.C. Greer, V. Gupta, S. Haller, A. Haselbeck, S.I. Hay, M. Holm, S. Hopkins, K.C. Iregbu, J. Jacobs, D. Jarovsky, F. Javanmardi, M. Khorana, N. Kissoon, E. Kobeissi, T. Kostyanév, F. Krapp, R. Krumkamp, A. Kumar, H.H. Kyu, C. Lim, D. Limmathurotsakul, M.J. Loftus, M. Lunn, J. Ma, N. Mturi, T. Munera-Huertas, P. Musicha, M.M. Mussi-Pinhata, T. Nakamura, R. Nanavati, S. Nangia, P. Newton, C. Ngoun, A. Novotney, D. Nwakanma, C.W. Obiero, A. Olivas-Martinez, P. Olliaro, E. Ooko, E. Ortiz-Brizuela, A.Y. Peleg, C. Perrone, N. Plakkal, A. Ponce-de-Leon, M. Raad, T. Ramdin, A. Riddell, T. Roberts, J.V. Robotham, A. Roca, K.E. Rudd, N. Russell, J. Schnall, J.A.G. Scott, M. Shivamallappa, J. Sifuentes-Osornio, N. Steenkeste, A.J. Stewardson, T. Stoeva, N. Tasak, A. Thaiprakong, G. Thwaites, C. Turner, P. Turner, H.R. van Doorn, S. Velaphi, A. Vongpradith, H. Vu, T. Walsh, S. Waner, T. Wangrangsimakul, T. Wozniak, P. Zheng, B. Sartorius, A.D. Lopez, A. Stergachis, C. Moore, C. Dolecek, M. Naghavi, Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis, *The Lancet* 399 (2022) 629–655. [https://doi.org/10.1016/S0140-6736\(21\)02724-0](https://doi.org/10.1016/S0140-6736(21)02724-0).
- [4] EClinicalMedicine, Antimicrobial resistance: a top ten global public health threat, *EClinicalMedicine* 41 (2021) 101221. <https://doi.org/10.1016/j.eclinm.2021.101221>.
- [5] G.D. Wright, Bacterial resistance to antibiotics: Enzymatic degradation and modification, *Adv Drug Deliv Rev* 57 (2005). <https://doi.org/10.1016/j.addr.2005.04.002>.
- [6] A. Zhao, J. Sun, Y. Liu, Understanding bacterial biofilms: From definition to treatment strategies, *Front Cell Infect Microbiol* 13 (2023) 1137947. <https://doi.org/10.3389/FCIMB.2023.1137947/BIBTEX>.
- [7] A.P. Kunnath, M.S. Suoodh, D. Kumar Chellappan, J. Chellian, K. Palaniveloo, Bacterial Persister Cells and Development of Antibiotic Resistance in Chronic Infections: An Update, (n.d.). <https://doi.org/10.3389/bjbs.2024.12958>.
- [8] J. Davies, Where Have all the Antibiotics Gone?, *Canadian Journal of Infectious Diseases and Medical Microbiology* 17 (2006) 287–290. <https://doi.org/10.1155/2006/707296>.
- [9] K.M.G. O’Connell, J.T. Hodgkinson, H.F. Sore, M. Welch, G.P.C. Salmond, D.R. Spring, Combating multidrug-resistant bacteria: Current strategies for the discovery of novel antibacterials, *Angewandte Chemie - International Edition* 52 (2013) 10706–10733. <https://doi.org/10.1002/ANIE.201209979>.
- [10] P. Fernandes, Antibacterial discovery and development—the failure of success?, *Nature Biotechnology* 2006 24:12 24 (2006) 1497–1503. <https://doi.org/10.1038/nbt1206-1497>.
- [11] U. Theuretzbacher, L.J.V. Piddock, Non-traditional Antibacterial Therapeutic Options and Challenges, *Cell Host Microbe* 26 (2019) 61–72. <https://doi.org/10.1016/J.CHOM.2019.06.004>.

- [12] R. Peraman, S.K. Sure, V.N.A. Dusthacker, N.B. Chilamakuru, P.R. Yiragamreddy, C. Pokuri, V.K. Kutagulla, S. Chinni, Insights on recent approaches in drug discovery strategies and untapped drug targets against drug resistance, *Future Journal of Pharmaceutical Sciences* 2021 7:1 7 (2021) 1–25. <https://doi.org/10.1186/S43094-021-00196-5>.
- [13] M. Gopikrishnan, S. Haryini, C. George Priya Doss, Emerging strategies and therapeutic innovations for combating drug resistance in *Staphylococcus aureus* strains: A comprehensive review, *J Basic Microbiol* 64 (2024) 2300579. <https://doi.org/10.1002/JOBM.202300579>.
- [14] C.R. MacNair, S.T. Rutherford, M.W. Tan, Alternative therapeutic strategies to treat antibiotic-resistant pathogens, *Nature Reviews Microbiology* 2023 22:5 22 (2023) 262–275. <https://doi.org/10.1038/s41579-023-00993-0>.
- [15] R. Thombre, S. Nazeer Khan, P. V Baptista, M. Martins mmartins, tedie R. Alexandra Fernandes, M.P. McCusker, A. Carvalho, D.A. Ferreira, N.M. Mohan, M. Martins, A.R. Fernandes, Nano-Strategies to Fight Multidrug Resistant Bacteria—`A Battle of the Titans`, *Frontiers in Microbiology | Www.Frontiersin.Org* 1 (2018) 1441. <https://doi.org/10.3389/fmicb.2018.01441>.
- [16] S. Kulshrestha, A.U. Khan, Nanomedicine for anticancer and antimicrobial treatment: an overview, *IET Nanobiotechnol* 12 (2018) 1009–1017. <https://doi.org/10.1049/IET-NBT.2018.5112>.
- [17] W. Zou, A. McAdorey, H. Yan, W. Chen, Nanomedicine to Overcome Antimicrobial Resistance: Challenges and Prospects, *Nanomedicine* 18 (2023) 471–484. <https://doi.org/10.2217/NNM-2023-0022>.
- [18] S. Das, P. Angsantikul, C. Le, D. Bao, Y. Miyamoto, W. Gao, L. Zhang, L. Eckmann, Neutralization of cholera toxin with nanoparticle decoys for treatment of cholera, *PLoS Negl Trop Dis* 12 (2018) e0006266. <https://doi.org/10.1371/JOURNAL.PNTD.0006266>.
- [19] E.J. Kwon, M. Skalak, A. Bertucci, G. Braun, F. Ricci, E. Ruoslahti, M.J. Sailor, S.N. Bhatia, Porous Silicon Nanoparticle Delivery of Tandem Peptide Anti-Infectives for the Treatment of *Pseudomonas aeruginosa* Lung Infections, *Advanced Materials* 29 (2017) 1701527. <https://doi.org/10.1002/ADMA.201701527>.
- [20] M. Thambirajoo, M. Maarof, Y. Lokanathan, H. Katas, N.F. Ghazalli, Y. Tabata, M.B. Fauzi, Potential of Nanoparticles Integrated with Antibacterial Properties in Preventing Biofilm and Antibiotic Resistance, *Antibiotics* 2021, Vol. 10, Page 1338 10 (2021) 1338. <https://doi.org/10.3390/ANTIBIOTICS10111338>.
- [21] P.P. Fu, Q. Xia, H.M. Hwang, P.C. Ray, H. Yu, Mechanisms of nanotoxicity: Generation of reactive oxygen species, *J Food Drug Anal* 22 (2014) 64–75. <https://doi.org/10.1016/J.JFDA.2014.01.005>.
- [22] Y. Huang, X. Guo, Y. Wu, X. Chen, L. Feng, N. Xie, G. Shen, Nanotechnology's frontier in combatting infectious and inflammatory diseases: prevention and treatment, *Signal Transduction and Targeted Therapy* 2024 9:1 9 (2024) 1–50. <https://doi.org/10.1038/s41392-024-01745-z>.
- [23] M. Wainwright, Photodynamic antimicrobial chemotherapy (PACT)., *Journal of Antimicrobial Chemotherapy* 42 (1998) 13–28. <https://doi.org/10.1093/jac/42.1.13>.
- [24] L.M. De Freitas, E.N. Lorenzón, N.A. Santos-Filho, L.H.D.P. Zago, M.P. Uliana, K.T. De Oliveira, E.M. Cilli, C.R. Fontana, Antimicrobial Photodynamic therapy enhanced by the peptide aurein, *Sci Rep* 8 (2018). <https://doi.org/10.1038/s41598-018-22687-x>.
- [25] X. Hu, Y.Y. Huang, Y. Wang, X. Wang, M.R. Hamblin, Antimicrobial photodynamic therapy to control clinically relevant biofilm infections, *Front Microbiol* 9 (2018) 377371. <https://doi.org/10.3389/FMICB.2018.01299/BIBTEX>.
- [26] H. Liu, F. Xing, Y. Zhou, P. Yu, J. Xu, R. Luo, Z. Xiang, P. Maria Rommens, M. Liu, U. Ritz, Nanomaterials-based photothermal therapies for antibacterial applications, *Mater Des* 233 (2023) 112231. <https://doi.org/10.1016/J.MATDES.2023.112231>.
- [27] G. Wu, Z. Xu, Y. Yu, M. Zhang, S. Wang, S. Duan, X. Liu, Biomaterials-based phototherapy for bacterial infections, *Front Pharmacol* 15 (2024) 1513850. <https://doi.org/10.3389/FPHAR.2024.1513850/BIBTEX>.
- [28] Y. Ye, Q. Zheng, Z. Wang, S. Wang, Z. Lu, Q. Chu, Y. Liu, K. Yao, B. Wei, H. Han, H. Chen, X. Zhang, Metal-phenolic nanoparticles enhance low temperature photothermal therapy for bacterial biofilm in superficial infections, *Journal of Nanobiotechnology* 22 (2024) 1–15. <https://doi.org/10.1186/S12951-024-02985-5/FIGURES/6>.

- [29] M. Overchuk, R.A. Weersink, B.C. Wilson, G. Zheng, Photodynamic and Photothermal Therapies: Synergy Opportunities for Nanomedicine, (2023). <https://doi.org/10.1021/acsnano.3c00891>.
- [30] P.M. Alves, C.C. Barrias, P. Gomes, M.C.L. Martins, Smart biomaterial-based systems for intrinsic stimuli-responsive chronic wound management, *Mater Today Chem* 22 (2021). <https://doi.org/10.1016/j.mtchem.2021.100623>.
- [31] Á. Serrano-Aroca, A. Cano-Vicent, R. Sabater i Serra, M. El-Tanani, A.A. Aljabali, M.M. Tambuwala, Y.K. Mishra, Scaffolds in the microbial resistant era: Fabrication, materials, properties and tissue engineering applications, *Mater Today Bio* 16 (2022) 100412. <https://doi.org/10.1016/J.MTBIO.2022.100412>.
- [32] Z. Aliakbar Ahovan, Z. Esmaeili, B.S. Eftekhari, S. Khosravimelal, M. Alehosseini, G. Orive, A. Dolatshahi-Pirouz, N. Pal Singh Chauhan, P.A. Janmey, A. Hashemi, S.C. Kundu, M. Gholipourmalekabadi, Antibacterial smart hydrogels: New hope for infectious wound management, *Mater Today Bio* 17 (2022) 100499. <https://doi.org/10.1016/J.MTBIO.2022.100499>.
- [33] V. Andreu, G. Mendoza, M. Arruebo, S. Irusta, Smart dressings based on nanostructured fibers containing natural origin antimicrobial, anti-inflammatory, and regenerative compounds, *Materials* 8 (2015). <https://doi.org/10.3390/ma8085154>.
- [34] H. Haidari, Z. Kopecki, A.T. Sutton, S. Garg, A.J. Cowin, K. Vasilev, Ph-responsive “smart” hydrogel for controlled delivery of silver nanoparticles to infected wounds, *Antibiotics* 10 (2021). <https://doi.org/10.3390/antibiotics10010049>.
- [35] M. V. Risbud, A.A. Hardikar, S. V. Bhat, R.R. Bhonde, pH-sensitive freeze-dried chitosan–polyvinyl pyrrolidone hydrogels as controlled release system for antibiotic delivery, *Journal of Controlled Release* 68 (2000) 23–30. [https://doi.org/10.1016/S0168-3659\(00\)00208-X](https://doi.org/10.1016/S0168-3659(00)00208-X).
- [36] Z. Lu, J. Zhang, Z. Yu, Q. Liu, K. Liu, M. Li, D. Wang, Hydrogel degradation triggered by pH for the smart release of antibiotics to combat bacterial infection, *New Journal of Chemistry* 41 (2017) 432–436. <https://doi.org/10.1039/C6NJ03260E>.
- [37] C. Tonda-Turo, F. Ruini, C. Ceresa, P. Gentile, P. Varela, A.M. Ferreira, L. Fracchia, G. Ciardelli, Nanostructured scaffold with biomimetic and antibacterial properties for wound healing produced by ‘green electrospinning,’ *Colloids Surf B Biointerfaces* 172 (2018) 233–243. <https://doi.org/10.1016/J.COLSURFB.2018.08.039>.
- [38] G. Rath, T. Hussain, G. Chauhan, T. Garg, A.K. Goyal, Development and characterization of cefazolin loaded zinc oxide nanoparticles composite gelatin nanofiber mats for postoperative surgical wounds, *Materials Science and Engineering C* 58 (2016) 242–253. <https://doi.org/10.1016/j.msec.2015.08.050>.
- [39] I. Santos Ferreira, J. Kikhney, L. Kursawe, S. Kasper, L.M.D. Gonçalves, A. Trampuz, A. Moter, A.F. Bettencourt, A.J. Almeida, Encapsulation in Polymeric Microparticles Improves Daptomycin Activity Against Mature Staphylococci Biofilms—a Thermal and Imaging Study, *AAPS PharmSciTech* 19 (2018). <https://doi.org/10.1208/s12249-018-0974-7>.
- [40] A. Dubey, H. Vahabi, V. Kumaravel, Antimicrobial and Biodegradable 3D Printed Scaffolds for Orthopedic Infections, *ACS Biomater Sci Eng* 9 (2023) 4020. <https://doi.org/10.1021/ACSBiomaterials.3c00115>.
- [41] F. Jin, H. Wang, Q. Li, C. Bai, Y. Zeng, G. Lai, S. Guo, X. Gu, W. Li, H. Zhang, Clinical application of photodynamic therapy for malignant airway tumors in China, *Thorac Cancer* 11 (2020) 181–190. <https://doi.org/10.1111/1759-7714.13223>.
- [42] T. Maisch, Resistance in antimicrobial photodynamic inactivation of bacteria, *Photochemical and Photobiological Sciences* 14 (2015). <https://doi.org/10.1039/c5pp00037h>.
- [43] B.P. George, A. Chota, P. Sarbadhikary, H. Abrahamse, Fundamentals and applications of metal nanoparticle-enhanced singlet oxygen generation for improved cancer photodynamic therapy, *Front Chem* 10 (2022) 964674. <https://doi.org/10.3389/FCHEM.2022.964674/BIBTEX>.
- [44] G.M.F. Calixto, J. Bernegossi, L.M. De Freitas, C.R. Fontana, M. Chorilli, A.M. Grumezescu, Nanotechnology-Based Drug Delivery Systems for Photodynamic Therapy of Cancer: A Review, *Molecules* 2016, Vol. 21, Page 342 21 (2016) 342. <https://doi.org/10.3390/MOLECULES21030342>.
- [45] D.H. Truong, P.T.T. Tran, T.H. Tran, Nanoparticles as carriers of photosensitizers to improve photodynamic therapy in cancer, *Pharm Dev Technol* 29 (2024) 221–235. <https://doi.org/10.1080/10837450.2024.2322570>.

- [46] S. Alfei, G.C. Schito, A.M. Schito, G. Zuccari, Reactive Oxygen Species (ROS)-Mediated Antibacterial Oxidative Therapies: Available Methods to Generate ROS and a Novel Option Proposal, *Int J Mol Sci* 25 (2024). <https://doi.org/10.3390/IJMS25137182>.
- [47] L. Huang, Y. Xuan, Y. Koide, T. Zhiyentayev, M. Tanaka, M.R. Hamblin, Type i and Type II mechanisms of antimicrobial photodynamic therapy: An in vitro study on gram-negative and gram-positive bacteria, *Lasers Surg Med* 44 (2012). <https://doi.org/10.1002/lsm.22045>.
- [48] M. Kim, H.Y. Jung, H.J. Park, Topical PDT in the Treatment of Benign Skin Diseases: Principles and New Applications, *Int J Mol Sci* 16 (2015) 23259. <https://doi.org/10.3390/IJMS161023259>.
- [49] L. Benov, Photodynamic therapy: current status and future directions, *Med Princ Pract* 24 Suppl 1 (2015) 14–28. <https://doi.org/10.1159/000362416>.
- [50] A. Almeida, M.A.F. Faustino, J.P.C. Tomé, Photodynamic inactivation of bacteria: finding the effective targets, *Future Med Chem* 7 (2015) 1221–1224. <https://doi.org/10.4155/fmc.15.59>.
- [51] D. Hu, Y. Deng, F. Jia, Q. Jin, J. Ji, Surface Charge Switchable Supramolecular Nanocarriers for Nitric Oxide Synergistic Photodynamic Eradication of Biofilms, *ACS Nano* 14 (2020). <https://doi.org/10.1021/acsnano.9b05493>.
- [52] G. McDonnell, A.D. Russell, Antiseptics and disinfectants: Activity, action, and resistance, *Clin Microbiol Rev* 12 (1999). <https://doi.org/10.1128/cmr.12.1.147>.
- [53] J.Y. Maillard, Bacterial target sites for biocide action, *J Appl Microbiol* 92 (2002) 16S-27S. <https://doi.org/10.1046/J.1365-2672.92.5S1.3.X>.
- [54] M. Wainwright, Dyes, flies, and sunny skies: photodynamic therapy and neglected tropical diseases, *Coloration Technology* 133 (2017) 3–14. <https://doi.org/https://doi.org/10.1111/cote.12259>.
- [55] J. Ghorbani, D. Rahban, S. Aghamiri, A. Teymouri, A. Bahador, Photosensitizers in antibacterial photodynamic therapy: an overview, *Laser Ther* 27 (2018) 293. https://doi.org/10.5978/ISLSM.27_18-RA-01.
- [56] H. Abrahamse, M.R. Hamblin, New photosensitizers for photodynamic therapy, *Biochemical Journal* 473 (2016). <https://doi.org/10.1042/BJ20150942>.
- [57] S. Parab, P.K. Achalla, N. Yanamandala, G. Singhvi, P. Kesharwani, S.K. Dubey, Sensitizers in photodynamic therapy, in: *Nanomaterials for Photodynamic Therapy*, 2023. <https://doi.org/10.1016/B978-0-323-85595-2.00002-5>.
- [58] D. Samaroo, E. Perez, A. Aggarwal, A. Wills, N. O'connor, Strategies for delivering porphyrinoid-based photosensitizers in therapeutic applications, *Ther Deliv* 5 (2014). <https://doi.org/10.4155/tde.14.46>.
- [59] N. Sekkat, H. Van Den Bergh, T. Nyokong, N. Lange, Like a bolt from the blue: Phthalocyanines in biomedical optics, *Molecules* 17 (2012). <https://doi.org/10.3390/molecules17010098>.
- [60] P. Sivakumar, M. Lee, Y.S. Kim, M.S. Shim, Photo-triggered antibacterial and anticancer activities of zinc oxide nanoparticles, *J Mater Chem B* 6 (2018). <https://doi.org/10.1039/c8tb00948a>.
- [61] D. Ziental, B. Czarczynska-Goslinska, D.T. Mlynarczyk, A. Glowacka-Sobotta, B. Stanisz, T. Goslinski, L. Sobotta, Titanium dioxide nanoparticles: Prospects and applications in medicine, *Nanomaterials* 10 (2020). <https://doi.org/10.3390/nano10020387>.
- [62] F. Vatansever, W.C.M.A. de Melo, P. Avci, D. Vecchio, M. Sadasivam, A. Gupta, R. Chandran, M. Karimi, N.A. Parizotto, R. Yin, G.P. Tegos, M.R. Hamblin, Antimicrobial strategies centered around reactive oxygen species - bactericidal antibiotics, photodynamic therapy, and beyond, *FEMS Microbiol Rev* 37 (2013). <https://doi.org/10.1111/1574-6976.12026>.
- [63] R. Langer, D.A. Tirrell, Designing materials for biology and medicine, *Nature* 2004 428:6982 428 (2004) 487–492. <https://doi.org/10.1038/nature02388>.
- [64] M. Vallet-Regí, Evolution of Biomaterials, *Front Mater* 9 (2022) 864016. <https://doi.org/10.3389/FMATS.2022.864016/BIBTEX>.
- [65] T.S. Santra, A.U.S. Shinde, eds., *Advanced Drug Delivery*, 26 (2023). <https://doi.org/10.1007/978-981-99-6564-9>.

- [66] L. Huang, C.J. Liu, Progress for the development of antibacterial surface based on surface modification technology, *Supramolecular Materials* 1 (2022) 100008. <https://doi.org/10.1016/J.SUPMAT.2022.100008>.
- [67] N.M. Alves, J.F. Mano, Chitosan derivatives obtained by chemical modifications for biomedical and environmental applications, *Int J Biol Macromol* 43 (2008) 401–414. <https://doi.org/10.1016/J.IJBIOMAC.2008.09.007>.
- [68] C. Chen, X.-W. Geng, Y.-H. Pan, Y.-N. Ma, Y.-X. Ma, S.-Z. Gao, X.-J. Huang, Synthesis and characterization of tannic acid-PEG hydrogel via Mitsunobu polymerization †, (2020). <https://doi.org/10.1039/c9ra09229c>.
- [69] D.A. Serov, A. V. Gritsaeva, F.M. Yanbaev, A. V. Simakin, S. V. Gudkov, Review of Antimicrobial Properties of Titanium Dioxide Nanoparticles, *International Journal of Molecular Sciences* 2024, Vol. 25, Page 10519 25 (2024) 10519. <https://doi.org/10.3390/IJMS251910519>.
- [70] S. Das, J.M. Dowding, K.E. Klump, J.F. McGinnis, W. Self, S. Seal, Cerium oxide nanoparticles: applications and prospects in nanomedicine, *Nanomedicine (Lond)* 8 (2013) 1483–1508. <https://doi.org/10.2217/NNM.13.133>.
- [71] Y. Lu, D. Cheng, B. Niu, X. Wang, X. Wu, A. Wang, Properties of Poly (Lactic-co-Glycolic Acid) and Progress of Poly (Lactic-co-Glycolic Acid)-Based Biodegradable Materials in Biomedical Research, *Pharmaceuticals* 16 (2023) 454. <https://doi.org/10.3390/PH16030454>.
- [72] S. Mondal, S. Park, J. Choi, T.T.H. Vu, V.H.M. Doan, T.T. Vo, B. Lee, J. Oh, Hydroxyapatite: A journey from biomaterials to advanced functional materials, *Adv Colloid Interface Sci* 321 (2023) 103013. <https://doi.org/10.1016/J.CIS.2023.103013>.
- [73] I. Negut, G. Dorcioman, V. Grumezescu, Scaffolds for Wound Healing Applications, *Polymers (Basel)* 12 (2020) 2010. <https://doi.org/10.3390/POLYM12092010>.
- [74] P. Song, D. Zhou, F. Wang, G. Li, L. Bai, J. Su, Programmable biomaterials for bone regeneration, *Mater Today Bio* 29 (2024) 101296. <https://doi.org/10.1016/J.MTBIO.2024.101296>.
- [75] Q. Tang, W. Wang, X. Zhang, X. Fang, C. Zhang, Z. Zhu, Y. Hou, M. Lai, Bi-functionalization of titanium with a mixture of peptides for improving its osteogenic and antibacterial activity, *Colloid Interface Sci Commun* 51 (2022) 100673. <https://doi.org/10.1016/J.COLCOM.2022.100673>.
- [76] P. Kuppusamy, M.M. Yusoff, G.P. Maniam, N. Govindan, Biosynthesis of metallic nanoparticles using plant derivatives and their new avenues in pharmacological applications – An updated report, *Saudi Pharmaceutical Journal* 24 (2016) 473–484. <https://doi.org/10.1016/J.JSPS.2014.11.013>.
- [77] M.C. Moulton, L.K. Braydich-Stolle, M.N. Nadagouda, S. Kunzelman, S.M. Hussain, R.S. Varma, Synthesis, characterization and biocompatibility of “green” synthesized silver nanoparticles using tea polyphenols, *Nanoscale* 2 (2010) 763–770. <https://doi.org/10.1039/C0NR00046A>.
- [78] S. Bochari, A. Zarepour, A. Kalantari-Hesari, F. Haghi, M.A. Shahbazi, A. Zarrabi, S. Taheri, A. Maleki, Injectable, antibacterial, and oxygen-releasing chitosan-based hydrogel for multimodal healing of bacteria-infected wounds, *J Mater Chem B* 11 (2023). <https://doi.org/10.1039/d3tb01278f>.
- [79] Y. Zhang, M. Zhang, D. Cheng, S. Xu, C. Du, L. Xie, W. Zhao, Applications of electrospun scaffolds with enlarged pores in tissue engineering, *Biomater Sci* 10 (2022) 1423–1447. <https://doi.org/10.1039/D1BM01651B>.
- [80] V. Gautam, R. Kumar, K. Das Manandhar, S.C. Kamble, eds., *Nanomedicine*, (2024). <https://doi.org/10.1007/978-3-031-72467-1>.
- [81] J. Marques, T.D. Gomes, M.A. Forte, R.F. Silva, C.J. Tavares, A new route for the synthesis of highly-active N-doped TiO₂ nanoparticles for visible light photocatalysis using urea as nitrogen precursor, *Catal Today* (2019). <https://doi.org/10.1016/j.cattod.2018.09.002>.
- [82] X. Wu, Applications of Titanium Dioxide Materials, in: *Titanium Dioxide - Advances and Applications*, 2022. <https://doi.org/10.5772/intechopen.99255>.
- [83] V. Ferrara, M. Marchetti, D. Alfieri, L. Targetti, M. Scopelliti, B. Pignataro, F. Pavone, V. Vetri, G. Sancataldo, Blue light activated photodegradation of biomacromolecules by N-doped titanium dioxide in a chitosan hydrogel matrix, *J Photochem Photobiol A Chem* 437 (2023). <https://doi.org/10.1016/j.jphotochem.2022.114451>.

- [84] N. Venkatachalam, M. Palanichamy, V. Murugesan, Sol-gel preparation and characterization of nanosize TiO₂: Its photocatalytic performance, *Mater Chem Phys* 104 (2007) 454–459. <https://doi.org/10.1016/J.MATCHEMPHYS.2007.04.003>.
- [85] T. Wen, J. Gao, J. Shen, Z. Zhou, Preparation and characterization of TiO₂ thin films by the sol-gel process, *Journal of Materials Science* 2001 36:24 36 (2001) 5923–5926. <https://doi.org/10.1023/A:1012989012840>.
- [86] S. Désilets, P. Brousseau, D. Chamberland, S. Singh, H. Feng, R. Turcotte, K. Armstrong, J. Anderson, Analyses of the thermal decomposition of urea nitrate at high temperature, *Thermochim Acta* 521 (2011) 59–65. <https://doi.org/10.1016/J.TCA.2011.04.004>.
- [87] S.A. Ansari, M.M. Khan, M.O. Ansari, M.H. Cho, Nitrogen-doped titanium dioxide (N-doped TiO₂) for visible light photocatalysis, *New Journal of Chemistry* 40 (2016) 3000–3009. <https://doi.org/10.1039/C5NJ03478G>.
- [88] A. Li Volsi, Nanosistemi polimerici con un core di oro colloidale per la veicolazione di farmaci antineoplastici ed imaging di tumori solidi, (2017). <https://iris.unipa.it/handle/10447/220697> (accessed October 11, 2024).
- [89] A.B. Serrano-Montes, D.J. De Aberasturi, J. Langer, J.J. Giner-Casares, L. Scarabelli, A. Herrero, L.M. Liz-Marzán, A General Method for Solvent Exchange of Plasmonic Nanoparticles and Self-Assembly into SERS-Active Monolayers, *Langmuir* 31 (2015) 9205–9213. https://doi.org/10.1021/ACS.LANGMUIR.5B01838/SUPPL_FILE/LA5B01838_SI_001.PDF.
- [90] M.M. Ghobashy, S.A. Alkhursani, H.A. Alqahtani, T.K. El-damhougy, M. Madani, Gold nanoparticles in microelectronics advancements and biomedical applications, *Materials Science and Engineering: B* 301 (2024) 117191. <https://doi.org/10.1016/J.MSEB.2024.117191>.
- [91] S. Link, M.B. Mohamed, M.A. El-Sayed, Simulation of the optical absorption spectra of gold nanorods as a function of their aspect ratio and the effect of the medium dielectric constant, *Journal of Physical Chemistry B* 103 (1999). <https://doi.org/10.1021/jp990183f>.
- [92] J. Becker, A. Trügler, A. Jakab, U. Hohenester, C. Sönnichsen, The optimal aspect ratio of gold nanorods for plasmonic bio-sensing, *Plasmonics* 5 (2010). <https://doi.org/10.1007/s11468-010-9130-2>.
- [93] P. Varvarà, L. Tranchina, G. Cavallaro, M. Licciardi, Preparation and characterization of gold nanorods coated with gellan gum and lipoic acid, *Applied Sciences (Switzerland)* 10 (2020). <https://doi.org/10.3390/app10238322>.
- [94] M. Licciardi, P. Varvarà, L. Tranchina, R. Puleio, L. Cicero, G. Cassata, G. Giammona, In vivo efficacy of verteporfin loaded gold nanorods for combined photothermal/photodynamic colon cancer therapy, *Int J Pharm* 625 (2022). <https://doi.org/10.1016/j.ijpharm.2022.122134>.
- [95] D.M. Alshangiti, M.M. Ghobashy, H.A. Alqahtani, T.K. El-Damhougy, M. Madani, The energetic and physical concept of gold nanorod-dependent fluorescence in cancer treatment and development of new photonic compounds|review, *RSC Adv* 13 (2023) 32223–32265. <https://doi.org/10.1039/D3RA05487J>.
- [96] R. Vasita, D.S. Katti, Nanofibers and their applications in tissue engineering, *Int J Nanomedicine* 1 (2006). <https://doi.org/10.2147/nano.2006.1.1.15>.
- [97] V. Beachley, X. Wen, Polymer nanofibrous structures: Fabrication, biofunctionalization, and cell interactions, *Progress in Polymer Science (Oxford)* 35 (2010). <https://doi.org/10.1016/j.progpolymsci.2010.03.003>.
- [98] Y.Z. Long, X. Yan, X.X. Wang, J. Zhang, M. Yu, Electrospinning: The Setup and Procedure, *Electrospinning: Nanofabrication and Applications* (2019) 21–52. <https://doi.org/10.1016/B978-0-323-51270-1.00002-9>.
- [99] M. Joshi, ed., *Nanotechnology in Textiles*, (2020). <https://doi.org/10.1201/9781003055815>.
- [100] *Handbook of Nanofibers*, *Handbook of Nanofibers* (2019). <https://doi.org/10.1007/978-3-319-53655-2>.
- [101] V. Beachley, X. Wen, Effect of electrospinning parameters on the nanofiber diameter and length, *Materials Science and Engineering C* 29 (2009). <https://doi.org/10.1016/j.msec.2008.10.037>.
- [102] S.A. Theron, E. Zussman, A.L. Yarin, Experimental investigation of the governing parameters in the electrospinning of polymer solutions, *Polymer (Guildf)* 45 (2004). <https://doi.org/10.1016/j.polymer.2004.01.024>.
- [103] R.S. Bhattarai, R.D. Bachu, S.H.S. Boddu, S. Bhaduri, Biomedical applications of electrospun nanofibers: Drug and nanoparticle delivery, *Pharmaceutics* 11 (2019). <https://doi.org/10.3390/pharmaceutics11010005>.

- [104] M.B. Stie, K. Kalouta, C.F.B. da Cunha, H.M. Feroze, V. Vetri, V. Foderà, Sustainable strategies for waterborne electrospinning of biocompatible nanofibers based on soy protein isolate, *Sustainable Materials and Technologies* 34 (2022). <https://doi.org/10.1016/j.susmat.2022.e00519>.
- [105] M.B. Stie, M. Corezzi, A.D. Juncos Bombin, F. Ajallouecian, E. Attrill, S. Pagliara, J. Jacobsen, I.S. Chronakis, H.M. Nielsen, V. Foderà, Waterborne Electrospinning of α -Lactalbumin Generates Tunable and Biocompatible Nanofibers for Drug Delivery, *ACS Appl Nano Mater* 3 (2020). <https://doi.org/10.1021/acsanm.9b02557>.
- [106] R. Krishnan, S. Sundarajan, S. Ramakrishna, Green processing of nanofibers for regenerative medicine, *Macromol Mater Eng* 298 (2013). <https://doi.org/10.1002/mame.201200323>.
- [107] E. Schoolaert, P. Ryckx, J. Geltmeyer, S. Maji, P.H.M. Van Steenberghe, D.R. D'Hooge, R. Hoogenboom, K. De Clerck, Waterborne Electrospinning of Poly(N-isopropylacrylamide) by Control of Environmental Parameters, *ACS Appl Mater Interfaces* 9 (2017) 24100–24110. https://doi.org/10.1021/ACSAMI.7B05074/SUPPL_FILE/AM7B05074_SI_001.PDF.
- [108] W. Zhou, X. Gong, Y. Li, Y. Si, S. Zhang, J. Yu, B. Ding, Environmentally friendly waterborne polyurethane nanofibrous membranes by emulsion electrospinning for waterproof and breathable textiles, *Chemical Engineering Journal* 427 (2022) 130925. <https://doi.org/10.1016/J.CEJ.2021.130925>.
- [109] M.B. Stie, K. Kalouta, C.F.B. da Cunha, H.M. Feroze, V. Vetri, V. Foderà, Sustainable strategies for waterborne electrospinning of biocompatible nanofibers based on soy protein isolate, *Sustainable Materials and Technologies* 34 (2022) e00519. <https://doi.org/10.1016/j.susmat.2022.e00519>.
- [110] D.N. Nguyen, C. Clasen, G. Van den Mooter, Pharmaceutical Applications of Electrospraying, *J Pharm Sci* 105 (2016) 2601–2620. <https://doi.org/10.1016/j.xphs.2016.04.024>.
- [111] A. Jaworek, Micro- and nanoparticle production by electrospraying, *Powder Technol* 176 (2007). <https://doi.org/10.1016/j.powtec.2007.01.035>.
- [112] A. Tanhaei, M. Mohammadi, H. Hamishehkar, M.R. Hamblin, Electrospraying as a novel method of particle engineering for drug delivery vehicles, *Journal of Controlled Release* 330 (2021) 851–865. <https://doi.org/10.1016/j.jconrel.2020.10.059>.
- [113] A. De Pieri, K. Ocorr, K. Jerreld, M. Lamoca, W. Hitzl, K. Wuertz-Kozak, Resveratrol Microencapsulation into Electrosprayed Polymeric Carriers for the Treatment of Chronic, Non-Healing Wounds, *Pharmaceutics* 14 (2022). <https://doi.org/10.3390/PHARMACEUTICS14040853>.
- [114] Y.H. Hsu, D.W.C. Chen, M.J. Li, Y.H. Yu, Y.C. Chou, S.J. Liu, Sustained delivery of analgesic and antimicrobial agents to knee joint by direct injections of electrosprayed multipharmaceutical-loaded nano/microparticles, *Polymers (Basel)* 10 (2018). <https://doi.org/10.3390/polym10080890>.
- [115] F. Schmid, Biological Macromolecules: UV-visible Spectrophotometry, in: *Encyclopedia of Life Sciences*, 2001. <https://doi.org/10.1038/npg.els.0003142>.
- [116] Z. Jia, J. Li, L. Gao, D. Yang, A. Kanaev, Dynamic Light Scattering: A Powerful Tool for In Situ Nanoparticle Sizing, *Colloids and Interfaces* 7 (2023). <https://doi.org/10.3390/colloids7010015>.
- [117] P.A. Hassan, S. Rana, G. Verma, Making sense of Brownian motion: Colloid characterization by dynamic light scattering, *Langmuir* 31 (2015) 3–12. https://doi.org/10.1021/LA501789Z/ASSET/IMAGES/LARGE/LA-2014-01789Z_0004.JPEG.
- [118] S. Bhattacharjee, DLS and zeta potential - What they are and what they are not?, *Journal of Controlled Release* 235 (2016). <https://doi.org/10.1016/j.jconrel.2016.06.017>.
- [119] S. Agnello, *Spectroscopy for Materials Characterization*, 2021. <https://doi.org/10.1002/9781119698029>.
- [120] M.M. Eid, Characterization of Nanoparticles by FTIR and FTIR-Microscopy, *Handbook of Consumer Nanoproducts* (2022) 645–673. https://doi.org/10.1007/978-981-16-8698-6_89.
- [121] J.R. Lakowicz, *Principles of fluorescence spectroscopy*, 2006. <https://doi.org/10.1007/978-0-387-46312-4>.
- [122] W. Denk, Two-photon excitation in functional biological imaging, <https://doi.org/10.1117/12.242945> 1 (1996) 296–304. <https://doi.org/10.1117/12.242945>.

- [123] A. Diaspro, G. Chirico, M. Collini, Two-photon fluorescence excitation and related techniques in biological microscopy, *Q Rev Biophys* 38 (2005). <https://doi.org/10.1017/S0033583505004129>.
- [124] P.T.C. So, C.Y. Dong, B.R. Masters, Two-Photon Excitation Fluorescence Microscopy, *Biomedical Photonics: Handbook* (2013) 2667–2671. https://doi.org/10.1007/978-3-642-16712-6_829.
- [125] P.T.C. So, C.Y. Dong, B.R. Masters, K.M. Berland, Two-photon excitation fluorescence microscopy, *Annu Rev Biomed Eng* 2 (2000) 399–429. <https://doi.org/10.1146/ANNUREV.BIOENG.2.1.399/CITE/REFWORKS>.
- [126] M.A. Digman, V.R. Caiolfa, M. Zamai, E. Gratton, The Phasor Approach to Fluorescence Lifetime Imaging Analysis, *Biophys J* 94 (2007) L14. <https://doi.org/10.1529/BIOPHYSJ.107.120154>.
- [127] M.A. Digman, V.R. Caiolfa, M. Zamai, E. Gratton, The phasor approach to fluorescence lifetime imaging analysis, *Biophys J* 94 (2008) L14–L16. <https://doi.org/10.1529/biophysj.107.120154>.
- [128] G. Weber, Resolution of the fluorescence lifetimes in a heterogeneous system by phase and modulation measurements, *Journal of Physical Chemistry* 85 (1981). <https://doi.org/10.1021/j150608a006>.
- [129] D.M. Jameson, E. Gratton, R.D. Hall, The measurement and analysis of heterogeneous emissions by multifrequency phase and modulation fluorometry, *Appl Spectrosc Rev* 20 (1984). <https://doi.org/10.1080/05704928408081716>.
- [130] S. Ranjit, L. Malacrida, D.M. Jameson, E. Gratton, Fit-free analysis of fluorescence lifetime imaging data using the phasor approach, *Nat Protoc* 13 (2018). <https://doi.org/10.1038/s41596-018-0026-5>.
- [131] G. De Luca, G. Sancataldo, B. Militello, V. Vetri, Surface-catalyzed liquid–liquid phase separation and amyloid-like assembly in microscale compartments, *J Colloid Interface Sci* 676 (2024) 569–581. <https://doi.org/10.1016/J.JCIS.2024.07.135>.
- [132] B. Torrado, B. Pannunzio, L. Malacrida, M.A. Digman, Fluorescence lifetime imaging microscopy, *Nature Reviews Methods Primers* 2024 4:1 4 (2024) 1–23. <https://doi.org/10.1038/s43586-024-00358-8>.
- [133] B. Torrado, B. Pannunzio, L. Malacrida, M.A. Digman, Fluorescence lifetime imaging microscopy, *Nature Reviews Methods Primers* 2024 4:1 4 (2024) 1–23. <https://doi.org/10.1038/s43586-024-00358-8>.
- [134] J. Willem Borst, A.J. W G Visser, C. Poudel, I. Mela, C.F. Kaminski, High-throughput, multi-parametric, and correlative fluorescence lifetime imaging, *Methods Appl Fluoresc* 8 (2020) 024005. <https://doi.org/10.1088/2050-6120/AB7364>.
- [135] S. Anselmo, T. Avola, K. Kalouta, S. Cataldo, G. Sancataldo, N. Muratore, V. Foderà, V. Vetri, A. Pettignano, Sustainable soy protein microsponges for efficient removal of lead (II) from aqueous environments, *Int J Biol Macromol* 239 (2023) 124276. <https://doi.org/10.1016/J.IJBIOMAC.2023.124276>.
- [136] E. Gratton, S. Breusegem, J.D.B. Sutin, Q. Ruan, N.P. Barry, Fluorescence lifetime imaging for the two-photon microscope: time-domain and frequency-domain methods, *Https://Doi.Org/10.1117/1.1586704* 8 (2003) 381–390. <https://doi.org/10.1117/1.1586704>.
- [137] B.R. Lentz, Use of fluorescent probes to monitor molecular order and motions within liposome bilayers, *Chem Phys Lipids* 64 (1993). [https://doi.org/10.1016/0009-3084\(93\)90060-G](https://doi.org/10.1016/0009-3084(93)90060-G).
- [138] P. Delcanale, S. Abbruzzetti, C. Viappiani, Photodynamic treatment of pathogens, *La Rivista Del Nuovo Cimento* 2022 45:6 45 (2022) 407–459. <https://doi.org/10.1007/S40766-022-00031-4>.
- [139] A. Kamkaew, S.H. Lim, H.B. Lee, L.V. Kiew, L.Y. Chung, K. Burgess, BODIPY dyes in photodynamic therapy, *Chem Soc Rev* 42 (2012) 77–88. <https://doi.org/10.1039/C2CS35216H>.
- [140] G.P.C. Drummen, B.M. Gadella, J.A. Post, J.F. Brouwers, Mass spectrometric characterization of the oxidation of the fluorescent lipid peroxidation reporter molecule C11-BODIPY581/591, *Free Radic Biol Med* 36 (2004). <https://doi.org/10.1016/j.freeradbiomed.2004.03.014>.
- [141] M.F. Poyton, A.M. Sendekci, X. Cong, P.S. Cremer, Cu 2+ Binds to Phosphatidylethanolamine and Increases Oxidation in Lipid Membranes, (2016). <https://doi.org/10.1021/jacs.5b11561>.
- [142] G.P.C. Drummen, L.C.M. Van Liebergen, J.A.F. Op den Kamp, J.A. Post, C11-BODIPY581/591, an oxidation-sensitive fluorescent lipid peroxidation probe: (Micro)spectroscopic characterization and validation of methodology, *Free Radic Biol Med* 33 (2002) 473–490. [https://doi.org/10.1016/S0891-5849\(02\)00848-1](https://doi.org/10.1016/S0891-5849(02)00848-1).

- [143] F. Wang, N. Naowarojna, Y. Zou, Stratifying ferroptosis sensitivity in cells and mouse tissues by photochemical activation of lipid peroxidation and fluorescent imaging, *STAR Protoc* 3 (2022) 101189. <https://doi.org/10.1016/j.xpro.2022.101189>.
- [144] J.C. Stockert, Lipid Peroxidation Assay Using BODIPY-Phenylbutadiene Probes: A Methodological Overview, *Methods in Molecular Biology* 2202 (2021) 199–214. https://doi.org/10.1007/978-1-0716-0896-8_16.
- [145] E. Bassan, A. Gualandi, P.G. Cozzi, P. Ceroni, Design of BODIPY dyes as triplet photosensitizers: electronic properties tailored for solar energy conversion, photoredox catalysis and photodynamic therapy, *Chem Sci* 12 (2021) 6607–6628. <https://doi.org/10.1039/D1SC00732G>.
- [146] G.M. Makrigiorgos, Detection of lipid peroxidation on erythrocytes using the excimer-forming property of a lipophilic BODIPY fluorescent dye, *J Biochem Biophys Methods* 35 (1997). [https://doi.org/10.1016/S0165-022X\(97\)00020-1](https://doi.org/10.1016/S0165-022X(97)00020-1).
- [147] A.J. Musser, S.K. Rajendran, K. Georgiou, L. Gai, R.T. Grant, Z. Shen, M. Cavazzini, A. Ruseckas, G.A. Turnbull, I.D.W. Samuel, J. Clark, D.G. Lidzey, Intermolecular states in organic dye dispersions: excimers vs. aggregates, *J Mater Chem C Mater* 5 (2017) 8380–8389. <https://doi.org/10.1039/C7TC02655B>.
- [148] M. Bacalum, M. Radu, S. Osella, S. Knippenberg, M. Ameloot, Generalized polarization and time-resolved fluorescence provide evidence for different populations of Laurdan in lipid vesicles, *J Photochem Photobiol B* 250 (2024) 112833. <https://doi.org/10.1016/J.JPHOTOBIOL.2023.112833>.
- [149] T. Parasassi, G. De Stasio, G. Ravagnan, R.M. Rusch, E. Gratton, Quantitation of lipid phases in phospholipid vesicles by the generalized polarization of Laurdan fluorescence, *Biophys J* 60 (1991). [https://doi.org/10.1016/S0006-3495\(91\)82041-0](https://doi.org/10.1016/S0006-3495(91)82041-0).
- [150] I. Levitan, Evaluating membrane structure by Laurdan imaging: Disruption of lipid packing by oxidized lipids, *Curr Top Membr* 88 (2021) 235–256. <https://doi.org/10.1016/BS.CTM.2021.10.003>.
- [151] Phospholipids Handbook, *Phospholipids Handbook* (2018). <https://doi.org/10.1201/9780203743577/PHOSPHOLIPIDS-HANDBOOK-GREGOR-CEVC>.
- [152] S.A. Sanchez, M.A. Tricerri, G. Gunther, E. Gratton, Laurdan Generalized Polarization: from cuvette to microscope, *Modern Research and Educational Topics in Microscopy* 2 (2007).
- [153] A. Mangiarotti, M. Siri, N.W. Tam, Z. Zhao, L. Malacrida, R. Dimova, Biomolecular condensates modulate membrane lipid packing and hydration, *Nature Communications* 2023 14:1 14 (2023) 1–19. <https://doi.org/10.1038/s41467-023-41709-5>.
- [154] T. Parasassi, E. Gratton, Membrane lipid domains and dynamics as detected by Laurdan fluorescence, *J Fluoresc* 5 (1995). <https://doi.org/10.1007/BF00718783>.
- [155] S. Anselmo, G. Sancataldo, C. Baiamonte, G. Pizzolanti, V. Vetri, Transportan 10 Induces Perturbation and Pores Formation in Giant Plasma Membrane Vesicles Derived from Cancer Liver Cells, *Biomolecules* 13 (2023). <https://doi.org/10.3390/biom13030492>.
- [156] S. Anselmo, G. Sancataldo, H. Mørck Nielsen, V. Foderà, V. Vetri, Peptide-Membrane Interactions Monitored by Fluorescence Lifetime Imaging: A Study Case of Transportan 10, *Langmuir* 37 (2021). <https://doi.org/10.1021/acs.langmuir.1c02392>.
- [157] L.A. Bagatolli, E. Gratton, Two photon fluorescence microscopy of coexisting lipid domains in giant unilamellar vesicles of binary phospholipid mixtures, *Biophys J* 78 (2000). [https://doi.org/10.1016/S0006-3495\(00\)76592-1](https://doi.org/10.1016/S0006-3495(00)76592-1).
- [158] C. Dietrich, L.A. Bagatolli, Z.N. Volovyk, N.L. Thompson, M. Levi, K. Jacobson, E. Gratton, Lipid rafts reconstituted in model membranes, *Biophys J* 80 (2001). [https://doi.org/10.1016/S0006-3495\(01\)76114-0](https://doi.org/10.1016/S0006-3495(01)76114-0).
- [159] A. Datye, A. DeLaRiva, Scanning Electron Microscopy (SEM), in: *Springer Handbooks*, 2023. https://doi.org/10.1007/978-3-031-07125-6_18.
- [160] N. Tanaka, *Scanning transmission electron microscopy of nanomaterials: Basics of imaging and analysis*, 2014. <https://doi.org/10.1142/P807>.
- [161] W. Zhou, Z.L. Wang, *Scanning microscopy for nanotechnology: Techniques and applications*, 2007. <https://doi.org/10.1007/978-0-387-39620-0>.

- [162] D.B. Williams, C.B. Carter, The Transmission Electron Microscope, *Transmission Electron Microscopy* (1996) 3–17. https://doi.org/10.1007/978-1-4757-2519-3_1.
- [163] M.L. Verma, B.S. Dhanya, Sukriti, V. Rani, M. Thakur, J. Jeslin, R. Kushwaha, Carbohydrate and protein based biopolymeric nanoparticles: Current status and biotechnological applications, *Int J Biol Macromol* 154 (2020) 390–412. <https://doi.org/10.1016/J.IJBIOMAC.2020.03.105>.
- [164] W.J. Baumgardner, Y. Yu, R. Hovden, S. Honrao, R.G. Hennig, H.D. Abruña, D. Muller, T. Hanrath, Nanoparticle metamorphosis: An in situ high-temperature transmission electron microscopy study of the structural evolution of heterogeneous Au:Fe₂O₃ nanoparticles, *ACS Nano* 8 (2014) 5315–5322. https://doi.org/10.1021/NN501543D/SUPPL_FILE/NN501543D_SI_004.PDF.
- [165] E.O. Ortega, H. Hosseinian, M.J. Rosales López, A. Rodríguez Vera, S. Hosseini, I. BA Meza, *Material Characterization Techniques and Applications*, 2022.
- [166] K.P. Menard, *Dynamic Mechanical Analysis: A Practical Introduction: Second Edition*, 2008.
- [167] F.P. Beer, R. Johnston, J. Dewolf, D. Mazurek, *Mechanics of Materials*, McGraw-Hill, 2013.
- [168] Stress–strain curve - Wikipedia, (n.d.). https://en.wikipedia.org/wiki/Stress%E2%80%93strain_curve (accessed January 20, 2025).
- [169] R.W. Hertzberg, R.Paul. Vinci, J.L.. Hertzberg, *Deformation and fracture mechanics of engineering materials*, (2021).
- [170] D. Lee, H. Zhang, S. Ryu, Elastic Modulus Measurement of Hydrogels, (2024). https://doi.org/10.1007/978-3-319-76573-0_60-1.
- [171] T.P. Weihs, S Hong, J.C. Bravman, W.D. Nix, Mechanical deflection of cantilever microbeams: A new technique for testing the mechanical properties of thin films, *J Mater Res* 3 (1988) 931–942. <https://doi.org/10.1557/JMR.1988.0931>.
- [172] W.O. Soboyejo, *Mechanical properties of engineered materials*, Marcel Dekker, 2003. <http://worldcat.org/oclc/300921090> (accessed January 20, 2025).
- [173] R. Sandström, Stress Strain Curves, in: *Springer Series in Materials Science*, 2024. https://doi.org/10.1007/978-3-031-49507-6_3.
- [174] N.M. Jukapli, S. Bagheri, Recent developments on titania nanoparticle as photocatalytic cancer cells treatment, *J Photochem Photobiol B* 163 (2016) 421–430. <https://doi.org/10.1016/j.jphotobiol.2016.08.046>.
- [175] M. Tomás-Gamasa, J.L. Mascareñas, TiO₂-Based Photocatalysis at the Interface with Biology and Biomedicine, *ChemBioChem* 21 (2020) 294–309. <https://doi.org/10.1002/CBIC.201900229>.
- [176] G. Sujatha, S. Shanthakumar, F. Chiampo, UV Light-Irradiated Photocatalytic Degradation of Coffee Processing Wastewater Using TiO₂ as a Catalyst, *Environments* 2020, Vol. 7, Page 47 7 (2020) 47. <https://doi.org/10.3390/ENVIRONMENTS7060047>.
- [177] S.M. Patil, S.P. Deshmukh, A.G. Dhodamani, K. V. More, S.D. Delekar, Different Strategies for Modification of Titanium Dioxide as Heterogeneous Catalyst in Chemical Transformations, *Curr Org Chem* 21 (2016) 821–833. <https://doi.org/10.2174/1385272820666161013151816>.
- [178] J. Xia, L. Dong, H. Song, J. Yang, X. Zhu, Preparation of doped TiO₂ nanomaterials and their applications in photocatalysis, *Bulletin of Materials Science* 46 (2023). <https://doi.org/10.1007/s12034-022-02847-6>.
- [179] A. Khlyustova, N. Sirotkin, T. Kusova, A. Kraev, V. Titov, A. Agafonov, Doped TiO₂: the effect of doping elements on photocatalytic activity, *Mater Adv* 1 (2020) 1193–1201. <https://doi.org/10.1039/D0MA00171F>.
- [180] D. Zhou, Y. Ito, Visible light-curable polymers for biomedical applications, *Sci China Chem* 57 (2014) 510–521. <https://doi.org/10.1007/s11426-014-5069-z>.
- [181] S. Rehman, R. Ullah, A.M. Butt, N.D. Gohar, Strategies of making TiO₂ and ZnO visible light active, *J Hazard Mater* 170 (2009). <https://doi.org/10.1016/j.jhazmat.2009.05.064>.
- [182] R. Asahi, T. Morikawa, Nitrogen complex species and its chemical nature in TiO₂ for visible-light sensitized photocatalysis, *Chem Phys* 339 (2007). <https://doi.org/10.1016/j.chemphys.2007.07.041>.

- [183] N.T. Nolan, D.W. Synnott, M.K. Seery, S.J. Hinder, A. Van Wassenhoven, S.C. Pillai, Effect of N-doping on the photocatalytic activity of sol-gel TiO₂, *J Hazard Mater* 211–212 (2012). <https://doi.org/10.1016/j.jhazmat.2011.08.074>.
- [184] X. Li, X. Chen, H. Niu, X. Han, T. Zhang, J. Liu, H. Lin, F. Qu, The synthesis of CdS/TiO₂ hetero-nanofibers with enhanced visible photocatalytic activity, *J Colloid Interface Sci* 452 (2015). <https://doi.org/10.1016/j.jcis.2015.04.034>.
- [185] G. Žerjav, J. Zavašnik, J. Kovač, A. Pintar, The influence of Schottky barrier height onto visible-light triggered photocatalytic activity of TiO₂ + Au composites, *Appl Surf Sci* 543 (2021). <https://doi.org/10.1016/j.apsusc.2020.148799>.
- [186] P. Ribao, J. Corredor, M.J. Rivero, I. Ortiz, Role of reactive oxygen species on the activity of noble metal-doped TiO₂ photocatalysts, *J Hazard Mater* (2019). <https://doi.org/10.1016/j.jhazmat.2018.05.026>.
- [187] T.C. Damato, C.C.S. De Oliveira, R.A. Ando, P.H.C. Camargo, A facile approach to TiO₂ colloidal spheres decorated with Au nanoparticles displaying well-defined sizes and uniform dispersion, *Langmuir* 29 (2013). <https://doi.org/10.1021/la3045219>.
- [188] K.L. Kelly, E. Coronado, L.L. Zhao, G.C. Schatz, The optical properties of metal nanoparticles: The influence of size, shape, and dielectric environment, *Journal of Physical Chemistry B* 107 (2003). <https://doi.org/10.1021/jp026731y>.
- [189] M. D’Acunto, P. Cioni, E. Gabellieri, G. Presciuttini, Exploiting gold nanoparticles for diagnosis and cancer treatments, *Nanotechnology* 32 (2021). <https://doi.org/10.1088/1361-6528/abe1ed>.
- [190] J. Zheng, X. Cheng, H. Zhang, X. Bai, R. Ai, L. Shao, J. Wang, Gold Nanorods: The Most Versatile Plasmonic Nanoparticles, *Chem Rev* 121 (2021) 13342–13453. <https://doi.org/10.1021/ACS.CHEMREV.1C00422>/ASSET/IMAGES/MEDIUM/CR1C00422_0030.GIF.
- [191] S. Link, M.A. El-Sayed, Spectral Properties and Relaxation Dynamics of Surface Plasmon Electronic Oscillations in Gold and Silver Nanodots and Nanorods, *Journal of Physical Chemistry B* 103 (1999). <https://doi.org/10.1021/jp9917648>.
- [192] L. Vigderman, B.P. Khanal, E.R. Zubarev, L. Vigderman, B.P. Khanal, E.R. Zubarev, Functional Gold Nanorods: Synthesis, Self-Assembly, and Sensing Applications, *Advanced Materials* 24 (2012) 4811–4841. <https://doi.org/10.1002/ADMA.201201690>.
- [193] M.M. Ghobashy, S.A. Alkhursani, H.A. Alqahtani, T.K. El-damhougy, M. Madani, Gold nanoparticles in microelectronics advancements and biomedical applications, *Materials Science and Engineering: B* 301 (2024) 117191. <https://doi.org/10.1016/J.MSEB.2024.117191>.
- [194] J. Lee, Y.H. Lee, C.B. Jeong, J.S. Choi, K.S. Chang, M. Yoon, Gold nanorods-conjugated TiO₂ nanoclusters for the synergistic combination of phototherapeutic treatments of cancer cells, *J Nanobiotechnology* 16 (2018). <https://doi.org/10.1186/s12951-018-0432-4>.
- [195] M.V. Dozzi, L. Prati, P. Canton, E. Selli, Effects of gold nanoparticles deposition on the photocatalytic activity of titanium dioxide under visible light, *Physical Chemistry Chemical Physics* 11 (2009). <https://doi.org/10.1039/b907317e>.
- [196] L. Khalil, S. Sabahat, W. Ahmed, Effect of Aspect Ratio on the Catalytic Activities of Gold Nanorods, *Catal Letters* 154 (2024). <https://doi.org/10.1007/s10562-023-04369-0>.
- [197] L. Cheng, D. Zhang, Y. Liao, F. Li, H. Zhang, Q. Xiang, Constructing functionalized plasmonic gold/titanium dioxide nanosheets with small gold nanoparticles for efficient photocatalytic hydrogen evolution, *J Colloid Interface Sci* 555 (2019). <https://doi.org/10.1016/j.jcis.2019.07.060>.
- [198] W. He, H. Huang, J. Yan, J. Zhu, Photocatalytic and antibacterial properties of Au-TiO₂ nanocomposite on monolayer graphene: From experiment to theory, *J Appl Phys* 114 (2013). <https://doi.org/10.1063/1.4836875>.
- [199] Y. Chen, Y. Wang, W. Li, Q. Yang, Q. Hou, L. Wei, L. Liu, F. Huang, M. Ju, Enhancement of photocatalytic performance with the use of noble-metal-decorated TiO₂ nanocrystals as highly active catalysts for aerobic oxidation under visible-light irradiation, *Appl Catal B* 210 (2017). <https://doi.org/10.1016/j.apcatb.2017.03.077>.

- [200] Y. Ma, X. Wang, Y. Jia, X. Chen, H. Han, C. Li, Titanium dioxide-based nanomaterials for photocatalytic fuel generations, *Chem Rev* 114 (2014) 9987–10043. https://doi.org/10.1021/CR500008U/SUPPL_FILE/CR500008U_SI_001.PDF.
- [201] V. Kumaravel, S. Mathew, J. Bartlett, S.C. Pillai, Photocatalytic hydrogen production using metal doped TiO₂: A review of recent advances, *Appl Catal B* 244 (2019). <https://doi.org/10.1016/j.apcatb.2018.11.080>.
- [202] D.A. Serov, A. V. Gritsaeva, F.M. Yanbaev, A. V. Simakin, S. V. Gudkov, Review of Antimicrobial Properties of Titanium Dioxide Nanoparticles, *International Journal of Molecular Sciences* 2024, Vol. 25, Page 10519 25 (2024) 10519. <https://doi.org/10.3390/IJMS251910519>.
- [203] K.S. Khashan, G.M. Sulaiman, F.A. Abdulameer, S. Albukhaty, M.A. Ibrahim, T. Al-Muhimeed, A.A. Alobaid, Antibacterial Activity of TiO₂ Nanoparticles Prepared by One-Step Laser Ablation in Liquid, *Applied Sciences* 2021, Vol. 11, Page 4623 11 (2021) 4623. <https://doi.org/10.3390/APP11104623>.
- [204] S. Bonetta, S. Bonetta, F. Motta, A. Strini, E. Carraro, Photocatalytic bacterial inactivation by TiO₂-coated surfaces, *AMB Express* 3 (2013) 1–8. <https://doi.org/10.1186/2191-0855-3-59/FIGURES/5>.
- [205] A.B. Younis, V. Milosavljevic, T. Fialova, K. Smerkova, H. Michalkova, P. Svec, P. Antal, P. Kopel, V. Adam, L. Zurek, K. Dolezelikova, Synthesis and characterization of TiO₂ nanoparticles combined with geraniol and their synergistic antibacterial activity, *BMC Microbiol* 23 (2023) 1–10. <https://doi.org/10.1186/S12866-023-02955-1/FIGURES/6>.
- [206] F. Hajareh Haghighi, M. Mercurio, S. Cerra, T.A. Salamone, R. Bianymotlagh, C. Palocci, V. Romano Spica, I. Fratoddi, Surface modification of TiO₂ nanoparticles with organic molecules and their biological applications, *J Mater Chem B* 11 (2023) 2334–2366. <https://doi.org/10.1039/D2TB02576K>.
- [207] T. An, H. Zhao, P.K. Wong, eds., *Advances in Photocatalytic Disinfection*, (2017). <https://doi.org/10.1007/978-3-662-53496-0>.
- [208] S. Goswami, R. Ghosh, P. Prasanthan, N. Kishore, Mode of interaction of altretamine with calf thymus DNA: biophysical insights, *J Biomol Struct Dyn* 41 (2023). <https://doi.org/10.1080/07391102.2022.2054472>.
- [209] I. Hussain, S. Fatima, S. Siddiqui, S. Ahmed, M. Tabish, Exploring the binding mechanism of β -resorcylic acid with calf thymus DNA: Insights from multi-spectroscopic, thermodynamic and bioinformatics approaches, *Spectrochim Acta A Mol Biomol Spectrosc* 260 (2021) 119952. <https://doi.org/10.1016/J.SAA.2021.119952>.
- [210] F. Salehi, H. Behboudi, G. Kavooosi, S.K. Ardestani, Oxidative DNA damage induced by ROS-modulating agents with the ability to target DNA: A comparison of the biological characteristics of citrus pectin and apple pectin, *Scientific Reports* 2018 8:1 8 (2018) 1–16. <https://doi.org/10.1038/s41598-018-32308-2>.
- [211] A.M. Chiorcea-Paquim, A.M. Oliveira-Brett, Nanostructured material-based electrochemical sensing of oxidative DNA damage biomarkers 8-oxoguanine and 8-oxodeoxyguanosine: a comprehensive review, *Microchimica Acta* 188 (2021). <https://doi.org/10.1007/S00604-020-04689-7>.
- [212] M.S. Cooke, M.D. Evans, M. Dizdaroglu, J. Lunec, Oxidative DNA damage: mechanisms, mutation, and disease, *The FASEB Journal* 17 (2003). <https://doi.org/10.1096/fj.02-0752rev>.
- [213] H. Xie, D. Yang, A. Heller, Z. Gao, Electrocatalytic oxidation of guanine, guanosine, and guanosine monophosphate, *Biophys J* 92 (2007). <https://doi.org/10.1529/biophysj.106.102632>.
- [214] A. Akbarzadeh, R. Rezaei-Sadabady, S. Davaran, S.W. Joo, N. Zarghami, Y. Hanifepour, M. Samiei, M. Kouhi, K. Nejati-Koshki, Liposome: Classification, preparation, and applications, *Nanoscale Res Lett* 8 (2013) 1–9. <https://doi.org/10.1186/1556-276X-8-102/TABLES/2>.
- [215] M.L. Manca, M. Aroffu, F. Fulgheri, M. Perra, I. Castangia, Conventional methods for preparing liposomes of various types (MLVs, LUVs, SUVs): What, where, how and when, *Liposomes in Drug Delivery: What, Where, How and When to Deliver* (2024) 461–488. <https://doi.org/10.1016/B978-0-443-15491-1.00011-0>.
- [216] T. Ohno, K. Sarukawa, K. Tokieda, M. Matsumura, Morphology of a TiO₂ photocatalyst (Degussa, P-25) consisting of anatase and rutile crystalline phases, *J Catal* 203 (2001). <https://doi.org/10.1006/jcat.2001.3316>.
- [217] S. Bock, C. Kijatkin, D. Berben, M. Imlau, Absorption and remission characterization of pure, dielectric (nano-)powders using diffuse reflectance spectroscopy: An end-to-end instruction, *Applied Sciences (Switzerland)* 9 (2019). <https://doi.org/10.3390/APP9224933>.

- [218] R. López, R. Gómez, Band-gap energy estimation from diffuse reflectance measurements on sol-gel and commercial TiO₂: A comparative study, *J Solgel Sci Technol* 61 (2012). <https://doi.org/10.1007/s10971-011-2582-9>.
- [219] S. Singh, S. Perween, A. Ranjan, Enhancing the visible light induced photocatalytic activity in sol-electrospun zinc titanate nanopowders by nitrogen doping, *Journal of Physics and Chemistry of Solids* 158 (2021) 110221. <https://doi.org/10.1016/J.JPCS.2021.110221>.
- [220] M. Raffi, J.I. Akhter, M.M. Hasan, Effect of annealing temperature on Ag nano-composite synthesized by sol-gel, *Mater Chem Phys* 99 (2006) 405–409. <https://doi.org/10.1016/J.MATCHEMPHYS.2005.11.012>.
- [221] M.L. Eggersdorfer, S.E. Pratsinis, Agglomerates and aggregates of nanoparticles made in the gas phase, *Advanced Powder Technology* 25 (2014) 71–90. <https://doi.org/10.1016/j.appt.2013.10.010>.
- [222] A. Teleki, R. Wengeler, L. Wengeler, H. Nirschl, S.E. Pratsinis, Distinguishing between aggregates and agglomerates of flame-made TiO₂ by high-pressure dispersion, *Powder Technol* 181 (2008) 292–300. <https://doi.org/10.1016/j.powtec.2007.05.016>.
- [223] Y. Won, K. Schwartzberg, K.A. Gray, TiO₂-based transparent coatings create self-cleaning surfaces, *Chemosphere* 208 (2018) 899–906. <https://doi.org/10.1016/J.CHEMOSPHERE.2018.06.014>.
- [224] Q. Maqbool, O. Favoni, T. Wicht, N. Lasemi, S. Sabbatini, M. Stöger-Pollach, M.L. Ruello, F. Tittarelli, G. Rupprechter, Highly Stable Self-Cleaning Paints Based on Waste-Valorized PNC-Doped TiO₂ Nanoparticles, *ACS Catalysis* 14 (2024) 4820–4834. https://doi.org/10.1021/ACSCATAL.3C06203/ASSET/IMAGES/LARGE/CS3C06203_0006.JPEG.
- [225] T. Kuckhoff, K. Landfester, K.A.I. Zhang, C.T.J. Ferguson, Photocatalytic Hydrogels with a High Transmission Polymer Network for Pollutant Remediation, *Chemistry of Materials* 33 (2021) 9131–9138. https://doi.org/10.1021/ACS.CHEMMATER.1C02180/ASSET/IMAGES/LARGE/CM1C02180_0004.JPEG.
- [226] A. Li Volsi, C. Scialabba, V. Vetri, G. Cavallaro, M. Licciardi, G. Giammona, Near-Infrared Light Responsive Folate Targeted Gold Nanorods for Combined Photothermal-Chemotherapy of Osteosarcoma, *ACS Appl Mater Interfaces* 9 (2017). <https://doi.org/10.1021/acsami.7b03711>.
- [227] J. Zheng, X. Cheng, H. Zhang, X. Bai, R. Ai, L. Shao, J. Wang, Gold Nanorods: The Most Versatile Plasmonic Nanoparticles, *Chem Rev* 121 (2021) 13342–13453. https://doi.org/10.1021/ACS.CHEMREV.1C00422/ASSET/IMAGES/MEDIUM/CR1C00422_0030.GIF.
- [228] V.M. Cepak, C.R. Martin, Preparation and stability of template-synthesized metal nanorod sols in organic solvents, *Journal of Physical Chemistry B* 102 (1998). <https://doi.org/10.1021/jp982882i>.
- [229] P. Singh, S. Roy, P. Sanpui, A. Banerjee, A. Jaiswal, Gold nanostructures for photothermal therapy, in: *Nanotechnology in Modern Animal Biotechnology: Recent Trends and Future Perspectives*, 2019. https://doi.org/10.1007/978-981-13-6004-6_2.
- [230] R. Kumar, L. Binetti, T.H. Nguyen, L.S.M. Alwis, A. Agrawal, T. Sun, K.T.V. Grattan, Determination of the Aspect-ratio Distribution of Gold Nanorods in a Colloidal Solution using UV-visible absorption spectroscopy, *Scientific Reports* 2019 9:1 9 (2019) 1–10. <https://doi.org/10.1038/s41598-019-53621-4>.
- [231] G. Giammona, S.E. Drago, G. Calabrese, P. Varvarà, M.G. Rizzo, N. Mauro, G. Nicotra, S. Conoci, G. Pitarresi, Galactosylated Polymer/Gold Nanorods Nanocomposites for Sustained and Pulsed Chemo-Photothermal Treatments of Hepatocarcinoma, *Pharmaceutics* 14 (2022). <https://doi.org/10.3390/pharmaceutics14112503>.
- [232] N. Bagheri, J. Hassanzadeh, Z.B. Al-Ruqeishi, N.S.A. Manan, H.A.J. Al Lawati, O.K. Abou-Zied, AuNRs attached TiO₂ NPs modified by cobalt-imidazolate frameworks as exceptional materials to improve the energy conversion efficiency of dye-sensitized solar cells, *Physical Chemistry Chemical Physics* 25 (2023) 19230–19238. <https://doi.org/10.1039/D3CP01576A>.
- [233] T.A. Nguyen, N.T.K. Nguyen, S. Van Vu, L.N. To, V.H. Lam, T.N.H. Lo, I. Park, T.H. Ho, K.Q. Vo, Controlled synthesis of gold nanorods and integrated with titanium dioxide for enhancing visible light-driven antioxidant activity, *Vietnam Journal of Chemistry* 61 (2023). <https://doi.org/10.1002/vjch.202300037>.
- [234] Y. Takahashi, N. Miyahara, S. Yamada, Gold nanorods embedded in titanium oxide film for sensing applications, *Analytical Sciences* 29 (2013). <https://doi.org/10.2116/analsci.29.101>.

- [235] Y.M.S. Jamil, M.A.H. Awad, H.M.A. Al-Maydama, A.N. Alhakimi, M.M.E. Shakhdofo, S.O. Mohammed, Gold nanoparticles loaded on TiO₂ nanoparticles doped with N₂ as an efficient electrocatalyst for glucose oxidation: preparation, characterization, and electrocatalytic properties, *J Anal Sci Technol* 13 (2022) 1–16. <https://doi.org/10.1186/S40543-022-00363-0/FIGURES/14>.
- [236] C. Fang, H. Jia, S. Chang, Q. Ruan, P. Wang, T. Chen, J. Wang, (Gold core)/(titania shell) nanostructures for plasmon-enhanced photon harvesting and generation of reactive oxygen species, *Energy Environ Sci* 7 (2014) 3431–3438. <https://doi.org/10.1039/C4EE01787K>.
- [237] M.C. Ortega-Liebana, J.L. Hueso, R. Arenal, J. Santamaria, Titania-coated gold nanorods with expanded photocatalytic response. Enzyme-like glucose oxidation under near-infrared illumination, *Nanoscale* 9 (2017) 1787–1792. <https://doi.org/10.1039/C6NR06300D>.
- [238] K.O. Iwuozor, J.O. Ighalo, E.C. Emenike, L.A. Ogunfowora, C.A. Igwegbe, Adsorption of methyl orange: A review on adsorbent performance, *Current Research in Green and Sustainable Chemistry* 4 (2021) 100179. <https://doi.org/10.1016/J.CRGSC.2021.100179>.
- [239] L. Wu, X. Liu, G. Lv, R. Zhu, L. Tian, M. Liu, Y. Li, W. Rao, T. Liu, L. Liao, Study on the adsorption properties of methyl orange by natural one-dimensional nano-mineral materials with different structures, *Scientific Reports* 2021 11:1 11 (2021) 1–11. <https://doi.org/10.1038/s41598-021-90235-1>.
- [240] M. Hou, F. Li, X. Liu, X. Wang, H. Wan, The effect of substituent groups on the reductive degradation of azo dyes by zerovalent iron, *J Hazard Mater* 145 (2007). <https://doi.org/10.1016/j.jhazmat.2006.11.019>.
- [241] J. Fan, Y. Guo, J. Wang, M. Fan, Rapid decolorization of azo dye methyl orange in aqueous solution by nanoscale zerovalent iron particles, *J Hazard Mater* 166 (2009). <https://doi.org/10.1016/j.jhazmat.2008.11.091>.
- [242] X.F. Lü, H.R. Ma, Q. Zhang, K. Du, Degradation of methyl orange by UV, O₃ and UV/O₃ systems: Analysis of the degradation effects and mineralization mechanism, *Research on Chemical Intermediates* 39 (2013) 4189–4203. <https://doi.org/10.1007/S11164-012-0935-9/FIGURES/10>.
- [243] C. Baiocchi, M.C. Brussino, E. Pramauro, A.B. Prevot, L. Palmisano, G. Marci, Characterization of methyl orange and its photocatalytic degradation products by HPLC/UV–VIS diode array and atmospheric pressure ionization quadrupole ion trap mass spectrometry, *Int J Mass Spectrom* 214 (2002) 247–256. [https://doi.org/10.1016/S1387-3806\(01\)00590-5](https://doi.org/10.1016/S1387-3806(01)00590-5).
- [244] C.H. Nguyen, C.C. Fu, R.S. Juang, Degradation of methylene blue and methyl orange by palladium-doped TiO₂ photocatalysis for water reuse: Efficiency and degradation pathways, *J Clean Prod* 202 (2018). <https://doi.org/10.1016/j.jclepro.2018.08.110>.
- [245] C. Baiocchi, M.C. Brussino, E. Pramauro, A.B. Prevot, L. Palmisano, G. Marci, Characterization of methyl orange and its photocatalytic degradation products by HPLC/UV–VIS diode array and atmospheric pressure ionization quadrupole ion trap mass spectrometry, *Int J Mass Spectrom* 214 (2002). [https://doi.org/10.1016/S1387-3806\(01\)00590-5](https://doi.org/10.1016/S1387-3806(01)00590-5).
- [246] J. Lee, Y.H. Lee, C.B. Jeong, J.S. Choi, K.S. Chang, M. Yoon, Gold nanorods-conjugated TiO₂ nanoclusters for the synergistic combination of phototherapeutic treatments of cancer cells, *J Nanobiotechnology* 16 (2018). <https://doi.org/10.1186/s12951-018-0432-4>.
- [247] Z. Duan, Y. Huang, D. Zhang, S. Chen, Electrospinning Fabricating Au/TiO₂ Network-like Nanofibers as Visible Light Activated Photocatalyst, *Scientific Reports* 2019 9:1 9 (2019) 1–8. <https://doi.org/10.1038/s41598-019-44422-w>.
- [248] M.C. Bunea, V.C. Diculescu, M. Enculescu, D. Oprea, T.A. Enache, Influence of the Photodegradation of Azathioprine on DNA and Cells, *International Journal of Molecular Sciences* 2022, Vol. 23, Page 14438 23 (2022) 14438. <https://doi.org/10.3390/IJMS232214438>.
- [249] L. Wang, G. Zhang, J. Pan, C. Xiong, D. Gong, Intercalation binding of food antioxidant butylated hydroxyanisole to calf thymus DNA, *J Photochem Photobiol B* 141 (2014) 253–261. <https://doi.org/10.1016/J.PHOTOBIO.2014.10.011>.
- [250] V. Muthuraj, M. Umadevi, Photo-degradation of CT-DNA with a series of carbothioamide ruthenium (II) complexes – Synthesis and structural analysis, *J Mol Struct* 1157 (2018) 201–209. <https://doi.org/10.1016/J.MOLSTRUC.2017.10.103>.

- [251] C. Wei, G. Jia, J. Yuan, Z. Feng, C. Li, A spectroscopic study on the interactions of porphyrin with G-quadruplex DNAs, *Biochemistry* 45 (2006). <https://doi.org/10.1021/bi052356z>.
- [252] S.O. Kelley, R.E. Holmlin, E.D.A. Stemp, J.K. Barton, Photoinduced electron transfer in ethidium-modified DNA duplexes: Dependence on distance and base stacking, *J Am Chem Soc* 119 (1997). <https://doi.org/10.1021/ja9714651>.
- [253] R.F. Pasternack, E.J. Gibbs, J.J. Villafranca, Interactions of Porphyrins with Nucleic Acids, *Biochemistry* 22 (1983). <https://doi.org/10.1021/bi00279a016>.
- [254] A.M. Chiorcea-Paquim, 8-oxoguanine and 8-oxodeoxyguanosine Biomarkers of Oxidative DNA Damage: A Review on HPLC–ECD Determination, *Molecules* 27 (2022). <https://doi.org/10.3390/MOLECULES27051620>.
- [255] T. Lindahl, Instability and decay of the primary structure of DNA, *Nature* 362 (1993) 709–715. <https://doi.org/10.1038/362709a0>.
- [256] R. Doshi, P.J.R. Day, P. Carampin, E. Blanch, I.J. Stratford, N. Tirelli, Spectrophotometric analysis of nucleic acids: Oxygenation-dependant hyperchromism of DNA, *Anal Bioanal Chem* 396 (2010) 2331–2339. <https://doi.org/10.1007/S00216-010-3461-X/FIGURES/7>.
- [257] N. Jayanth, S. Ramachandran, M. Puranik, Solution structure of the DNA damage lesion 8-oxoguanosine from ultraviolet resonance Raman Spectroscopy, *Journal of Physical Chemistry A* 113 (2009) 1459–1471. https://doi.org/10.1021/JP8071519/SUPPL_FILE/JP8071519_SI_001.PDF.
- [258] N. Kasyanenko, V. Bakulev, I. Perevyazko, T. Nekrasova, O. Nazarova, A. Slita, Y. Zolotova, E. Panarin, Model system for multifunctional delivery nanoplatfroms based on DNA-Polymer complexes containing silver nanoparticles and fluorescent dye, *J Biotechnol* 236 (2016). <https://doi.org/10.1016/j.jbiotec.2016.08.010>.
- [259] C.M.C. Andrés, J.M.P. de la Lastra, C.A. Juan, F.J. Plou, E. Pérez-Lebeña, Chemical Insights into Oxidative and Nitrate Modifications of DNA, *Int J Mol Sci* 24 (2023). <https://doi.org/10.3390/IJMS242015240>.
- [260] K.T. Karthikeyan, A. Nithya, K. Jothivenkatachalam, Photocatalytic and antimicrobial activities of chitosan-TiO₂ nanocomposite, *Int J Biol Macromol* 104 (2017) 1762–1773. <https://doi.org/10.1016/J.IJBIOMAC.2017.03.121>.
- [261] A.N. Severyukhina, N. V. Petrova, K. Smuda, G.S. Terentyuk, B.N. Klebtsov, R. Georgieva, H. Bäuml, D.A. Gorin, Photosensitizer-loaded electrospun chitosan-based scaffolds for photodynamic therapy and tissue engineering, *Colloids Surf B Biointerfaces* 144 (2016). <https://doi.org/10.1016/j.colsurfb.2016.03.081>.
- [262] L.M. Bravo-Anaya, J.F.A. Soltero, M. Rinaudo, DNA/chitosan electrostatic complex, *Int J Biol Macromol* 88 (2016) 345–353. <https://doi.org/10.1016/J.IJBIOMAC.2016.03.035>.
- [263] S.P. Strand, S. Danielsen, B.E. Christensen, K.M. Vårum, Influence of chitosan structure on the formation and stability of DNA-chitosan polyelectrolyte complexes, *Biomacromolecules* 6 (2005) 3357–3366. <https://doi.org/10.1021/BM0503726/ASSET/IMAGES/LARGE/BM0503726F00009.JPEG>.
- [264] W. Liao, M. Zhao, H. Rong, P. Jiang, Q. Liao, C. Zhang, Y. Chen, Photocatalyst immobilized by hydrogel, efficient degradation and self regeneration: A review, *Mater Sci Semicond Process* 150 (2022) 106929. <https://doi.org/10.1016/J.MSSP.2022.106929>.
- [265] O.K. Dalrymple, E. Stefanakos, M.A. Trotz, D.Y. Goswami, A review of the mechanisms and modeling of photocatalytic disinfection, *Appl Catal B* 98 (2010). <https://doi.org/10.1016/j.apcatb.2010.05.001>.
- [266] M. Premanathan, K. Karthikeyan, K. Jeyasubramanian, G. Manivannan, Selective toxicity of ZnO nanoparticles toward Gram-positive bacteria and cancer cells by apoptosis through lipid peroxidation, *Nanomedicine* 7 (2011). <https://doi.org/10.1016/j.nano.2010.10.001>.
- [267] O.K. Dalrymple, W. Isaacs, E. Stefanakos, M.A. Trotz, D.Y. Goswami, Lipid vesicles as model membranes in photocatalytic disinfection studies, *J Photochem Photobiol A Chem* 221 (2011). <https://doi.org/10.1016/j.jphotochem.2011.04.025>.
- [268] E. Schnitzer, I. Pinchuk, D. Lichtenberg, Peroxidation of liposomal lipids, *European Biophysics Journal* 36 (2007) 499–515. <https://doi.org/10.1007/S00249-007-0146-2/FIGURES/2>.
- [269] E. Parra-Ortiz, L. Caselli, M. Agnoletti, M.W.A. Skoda, X. Li, D. Zhao, M. Malmsten, Mesoporous silica as a matrix for photocatalytic titanium dioxide nanoparticles: lipid membrane interactions, *Nanoscale* 14 (2022) 12297–12312. <https://doi.org/10.1039/D2NR01958B>.

- [270] L. Caselli, E. Parra-Ortiz, S. Micciulla, M.W.A. Skoda, S.M. Häffner, E.M. Nielsen, M.J.A. van der Plas, M. Malmsten, Boosting Membrane Interactions and Antimicrobial Effects of Photocatalytic Titanium Dioxide Nanoparticles by Peptide Coating, *Small* 20 (2024). <https://doi.org/10.1002/sml.202309496>.
- [271] J. Kiwi, V. Nadtochenko, New evidence for TiO₂ photocatalysis during bilayer lipid peroxidation, *Journal of Physical Chemistry B* 108 (2004). <https://doi.org/10.1021/jp048281a>.
- [272] E. Parra-Ortiz, K.L. Browning, L.S.E. Damgaard, R. Nordström, S. Micciulla, S. Bucciarelli, M. Malmsten, Effects of oxidation on the physicochemical properties of polyunsaturated lipid membranes, *J Colloid Interface Sci* 538 (2019) 404–419. <https://doi.org/10.1016/J.JCIS.2018.12.007>.
- [273] J.C. Stockert, Lipid peroxidation assay using bodipy-phenylbutadiene probes: A methodological overview, in: *Methods in Molecular Biology*, 2021. https://doi.org/10.1007/978-1-0716-0896-8_16.
- [274] G. Cheloni, V.I. Slaveykova, Optimization of the C11-BODIPY581/591 dye for the determination of lipid oxidation in *Chlamydomonas reinhardtii* by flow cytometry, *Cytometry Part A* 83 (2013) 952–961. <https://doi.org/10.1002/CYTO.A.22338>.
- [275] T.D. Cummins, A.N. Higdon, P.A. Kramer, B.K. Chacko, D.W. Riggs, J.K. Salabei, L.J. Dell'Italia, J. Zhang, V.M. Darley-Usmar, B.G. Hill, Utilization of fluorescent probes for the quantification and identification of subcellular proteomes and biological processes regulated by lipid peroxidation products, *Free Radic Biol Med* 59 (2013) 56–68. <https://doi.org/10.1016/J.FREERADBIOMED.2012.08.014>.
- [276] L. Quan, W. Lin, T. Sun, Z. Xie, Y. Huang, X. Jing, Green photocatalysis with oxygen sensitive BODIPYs under visible light, *Catal Letters* 144 (2014) 308–313. <https://doi.org/10.1007/S10562-013-1122-3/FIGURES/5>.
- [277] S. Mazeres, E. Joly, A. Lopez, C. Tardin, Characterization of M-laurdan, a versatile probe to explore order in lipid membranes, *F1000Res* 3 (2014). <https://doi.org/10.12688/f1000research.4805.2>.
- [278] G. Gunther, L. Malacrida, D.M. Jameson, E. Gratton, S.A. Sánchez, LAURDAN since Weber: The Quest for Visualizing Membrane Heterogeneity, *Acc Chem Res* 54 (2021). <https://doi.org/10.1021/acs.accounts.0c00687>.
- [279] T. Parasassi, G. De Stasio, A. d'Ubaldo, E. Gratton, Phase fluctuation in phospholipid membranes revealed by Laurdan fluorescence, *Biophys J* 57 (1990). [https://doi.org/10.1016/S0006-3495\(90\)82637-0](https://doi.org/10.1016/S0006-3495(90)82637-0).
- [280] M.A. Soto-Arriaza, C.P. Sotomayor, E.A. Lissi, Relationship between lipid peroxidation and rigidity in L- α -phosphatidylcholine-DPPC vesicles, *J Colloid Interface Sci* 323 (2008). <https://doi.org/10.1016/j.jcis.2008.04.034>.
- [281] T. Hendel, M. Wuthschick, F. Kettemann, A. Birnbaum, K. Rademann, J. Polte, In situ determination of colloidal gold concentrations with uv-vis spectroscopy: Limitations and perspectives, *Anal Chem* 86 (2014) 11115–11124. https://doi.org/10.1021/AC502053S/SUPPL_FILE/AC502053S_SI_001.PDF.
- [282] W.W. Wilfinger, K. Mackey, P. Chomczynski, NanoDrop and design are registered trademarks of NanoDrop Technologies 260/280 and 260/230 Ratios NanoDrop® ND-1000 and ND-8000 8-Sample Spectrophotometers, *Biotechniques* 22 (1997).
- [283] E. Rideau, R. Dimova, P. Schwille, F.R. Wurm, K. Landfester, Liposomes and polymersomes: a comparative review towards cell mimicking, *Chem Soc Rev* 47 (2018) 8572–8610. <https://doi.org/10.1039/C8CS00162F>.
- [284] F. Olson, C.A. Hunt, F.C. Szoka, W.J. Vail, D. Papahadjopoulos, Preparation of liposomes of defined size distribution by extrusion through polycarbonate membranes, *Biochimica et Biophysica Acta (BBA) - Biomembranes* 557 (1979) 9–23. [https://doi.org/10.1016/0005-2736\(79\)90085-3](https://doi.org/10.1016/0005-2736(79)90085-3).
- [285] Laurdan | Fluorescent Lipids Probes and Cell Membrane Stains: R&D Systems, (n.d.). https://www.rndsystems.com/products/laurdan_7275 (accessed November 23, 2024).
- [286] F.M. Harris, K.B. Best, J.D. Bell, Use of laurdan fluorescence intensity and polarization to distinguish between changes in membrane fluidity and phospholipid order, *Biochim Biophys Acta Biomembr* 1565 (2002). [https://doi.org/10.1016/S0005-2736\(02\)00514-X](https://doi.org/10.1016/S0005-2736(02)00514-X).
- [287] T. Parasassi, E.K. Krasnowska, L. Bagatolli, E. Gratton, Laurdan and Prodan as Polarity-Sensitive Fluorescent Membrane Probes, *J Fluoresc* 8 (1998). <https://doi.org/10.1023/A:1020528716621>.
- [288] W. Thielemans, E. Can, S.S. Morye, R.P. Wool, Novel applications of lignin in composite materials, *J Appl Polym Sci* 83 (2002). <https://doi.org/10.1002/app.2247>.

- [289] K. Shi, G. Liu, H. Sun, Y. Weng, Poly(lactic Acid)/Lignin Composites: A Review, *Polymers (Basel)* 15 (2023). <https://doi.org/10.3390/polym15132807>.
- [290] H. Wang, X. Chen, L. Zhang, Z. Li, X. Fan, S. Sun, Efficient production of lignin-based slow-release nitrogen fertilizer via microwave heating, *Ind Crops Prod* 166 (2021). <https://doi.org/10.1016/j.indcrop.2021.113481>.
- [291] M. Ma, L. Dai, J. Xu, Z. Liu, Y. Ni, A simple and effective approach to fabricate lignin nanoparticles with tunable sizes based on lignin fractionation, *Green Chemistry* 22 (2020). <https://doi.org/10.1039/d0gc00377h>.
- [292] A. Khan, V. Nair, J.C. Colmenares, R. Gläser, Lignin-Based Composite Materials for Photocatalysis and Photovoltaics, *Top Curr Chem* 376 (2018) 1–31. <https://doi.org/10.1007/S41061-018-0198-Z/METRICS>.
- [293] S. Perveen, R. Zhai, Y. Zhang, M. Kawish, M.R. Shah, S. Chen, Z. Xu, D. Qiufeng, M. Jin, Boosting photo-induced antimicrobial activity of lignin nanoparticles with curcumin and zinc oxide, *Int J Biol Macromol* 253 (2023) 127433. <https://doi.org/10.1016/J.IJBIOMAC.2023.127433>.
- [294] H. Wang, Y. Pu, A. Ragauskas, B. Yang, From lignin to valuable products—strategies, challenges, and prospects, *Bioresour Technol* 271 (2019) 449–461. <https://doi.org/10.1016/J.BIORTECH.2018.09.072>.
- [295] R.C. Sun, Lignin Source and Structural Characterization, *ChemSusChem* 13 (2020) 4385–4393. <https://doi.org/10.1002/CSSC.202001324>.
- [296] Y.-C. Lu, Y. Lu, X. Fan, Structure and Characteristics of Lignin, (2020) 17–75. https://doi.org/10.1007/978-3-030-40663-9_2.
- [297] J.N.G. Stanley, M. Selva, A.F. Masters, T. Maschmeyer, A. Perosa, Reactions of p-coumaryl alcohol model compounds with dimethyl carbonate. Towards the upgrading of lignin building blocks, *Green Chemistry* 15 (2013) 3195–3204. <https://doi.org/10.1039/C3GC40334C>.
- [298] D. Ekeberg, K.S. Gretland, J. Gustafsson, S.M. Bråten, G.E. Fredheim, Characterisation of lignosulphonates and kraft lignin by hydrophobic interaction chromatography, *Anal Chim Acta* 565 (2006) 121–128. <https://doi.org/10.1016/J.ACA.2006.02.008>.
- [299] J. Rumpf, X.T. Do, R. Burger, Y. Monakhova, M. Schulze, Types of lignin, properties, and structural characterization techniques, *Lignin-Based Materials for Biomedical Applications: Preparation, Characterization, and Implementation* (2021) 105–158. <https://doi.org/10.1016/B978-0-12-820303-3.00001-1>.
- [300] H. Wang, Y. Pu, A. Ragauskas, B. Yang, From lignin to valuable products—strategies, challenges, and prospects, *Bioresour Technol* 271 (2019) 449–461. <https://doi.org/10.1016/J.BIORTECH.2018.09.072>.
- [301] E. Lizundia, M.H. Sipponen, L.G. Greca, M. Balakshin, B.L. Tardy, O.J. Rojas, D. Puglia, Multifunctional lignin-based nanocomposites and nanohybrids, *Green Chemistry* 23 (2021). <https://doi.org/10.1039/d1gc01684a>.
- [302] P. Figueiredo, K. Lintinen, J.T. Hirvonen, M.A. Kostianen, H.A. Santos, Properties and chemical modifications of lignin: Towards lignin-based nanomaterials for biomedical applications, *Prog Mater Sci* 93 (2018). <https://doi.org/10.1016/j.pmatsci.2017.12.001>.
- [303] J. Sameni, S. Krigstin, M. Sain, Solubility of Lignin and Acetylated Lignin in Organic Solvents, *Bioresources* 12 (2017). <https://doi.org/10.15376/biores.12.1.1548-1565>.
- [304] E. Melro, L. Alves, F.E. Antunes, B. Medronho, A brief overview on lignin dissolution, *J Mol Liq* 265 (2018). <https://doi.org/10.1016/j.molliq.2018.06.021>.
- [305] D. Kai, K. Zhang, L. Jiang, H.Z. Wong, Z. Li, Z. Zhang, X.J. Loh, Sustainable and Antioxidant Lignin-Polyester Copolymers and Nanofibers for Potential Healthcare Applications, *ACS Sustain Chem Eng* 5 (2017). <https://doi.org/10.1021/acssuschemeng.7b00850>.
- [306] H.A. Santos, P. Figueiredo, *Lignin-Based Materials for Biomedical Applications: Preparation, Characterization, and Implementation*, 2021. <https://doi.org/10.1016/B978-0-12-820303-3.00021-7>.
- [307] L. García-Fuentevilla, J.F. Rubio-Valle, R. Martín-Sampedro, C. Valencia, M.E. Eugenio, D. Ibarra, Different Kraft lignin sources for electrospun nanostructures production: Influence of chemical structure and composition, *Int J Biol Macromol* 214 (2022) 554–567. <https://doi.org/10.1016/J.IJBIOMAC.2022.06.121>.
- [308] D. Kai, S. Jiang, Z.W. Low, X.J. Loh, Engineering highly stretchable lignin-based electrospun nanofibers for potential biomedical applications, *J Mater Chem B* 3 (2015) 6194–6204. <https://doi.org/10.1039/C5TB00765H>.

- [309] D. Kai, W. Ren, L. Tian, P.L. Chee, Y. Liu, S. Ramakrishna, X.J. Loh, Engineering Poly(lactide)-Lignin Nanofibers with Antioxidant Activity for Biomedical Application, *ACS Sustain Chem Eng* 4 (2016). <https://doi.org/10.1021/acsschemeng.6b00478>.
- [310] C.Z. Mosher, P.A.P. Brudnicki, Z. Gong, H.R. Childs, S.W. Lee, R.M. Antrobus, E.C. Fang, T.N. Schiros, H.H. Lu, Green electrospinning for biomaterials and biofabrication, *Biofabrication* 13 (2021). <https://doi.org/10.1088/1758-5090/AC0964>.
- [311] J. Avossa, G. Herwig, C. Toncelli, F. Itel, R.M. Rossi, Electrospinning based on benign solvents: current definitions, implications and strategies, *Green Chemistry* 24 (2022) 2347–2375. <https://doi.org/10.1039/D1GC04252A>.
- [312] A. Malara, Environmental concerns on the use of the electrospinning technique for the production of polymeric micro/nanofibers, *Scientific Reports* 2024 14:1 14 (2024) 1–11. <https://doi.org/10.1038/s41598-024-58936-5>.
- [313] J. Wu, M. Wang, L. Dong, C. Zhu, J. Shi, H. Morikawa, Ultraflexible, Breathable, and Form-Stable Phase Change Fibrous Membranes by Green Electrospinning for Personal Thermal Management, *ACS Sustain Chem Eng* 10 (2022) 7873–7882. https://doi.org/10.1021/ACSSUSCHEMENG.2C00189/ASSET/IMAGES/LARGE/SC2C00189_0006.JPEG.
- [314] V. Poursorkhabi, A.K. Mohanty, M. Misra, Electrospinning of aqueous lignin/poly(ethylene oxide) complexes, *J Appl Polym Sci* 132 (2015). <https://doi.org/10.1002/app.41260>.
- [315] C.R. Kruse, M. Singh, S. Targosinski, I. Sinha, J.A. Sørensen, E. Eriksson, K. Nuutila, The effect of pH on cell viability, cell migration, cell proliferation, wound closure, and wound reepithelialization: In vitro and in vivo study, *Wound Repair and Regeneration* 25 (2017) 260–269. <https://doi.org/10.1111/WRR.12526>.
- [316] P. Karagoz, S. Khiawjan, M.P.C. Marques, S. Santzouk, T.D.H. Bugg, G.J. Lye, Pharmaceutical applications of lignin-derived chemicals and lignin-based materials: linking lignin source and processing with clinical indication, *Biomass Conversion and Biorefinery* 2023 14:21 14 (2023) 26553–26574. <https://doi.org/10.1007/S13399-023-03745-5>.
- [317] J. Zhang, Z. Tian, X. Ji, F. Zhang, Fabrication Mechanisms of Lignin Nanoparticles and Their Ultraviolet Protection Ability in PVA Composite Film, *Polymers (Basel)* 14 (2022). <https://doi.org/10.3390/polym14194196>.
- [318] Y. Zheng, A. Moreno, Y. Zhang, M.H. Sipponen, L. Dai, Harnessing chemical functionality of lignin towards stimuli-responsive materials, *Trends Chem* 6 (2024) 62–78. <https://doi.org/10.1016/J.TRECHM.2023.12.001>.
- [319] S. Sugiarto, Y. Leow, C.L. Tan, G. Wang, D. Kai, How far is Lignin from being a biomedical material?, *Bioact Mater* 8 (2022) 71–94. <https://doi.org/10.1016/J.BIOACTMAT.2021.06.023>.
- [320] E. Melro, A. Filipe, D. Sousa, A.J.M. Valente, A. Romano, F.E. Antunes, B. Medronho, Dissolution of kraft lignin in alkaline solutions, (2020). <https://doi.org/10.1016/j.ijbiomac.2020.01.153>.
- [321] S. Constant, H.L.J. Wienk, A.E. Frissen, P. De Peinder, R. Boelens, D.S. Van Es, R.J.H. Grisel, B.M. Weckhuysen, W.J.J. Huijgen, R.J.A. Gosselink, P.C.A. Bruijninx, New insights into the structure and composition of technical lignins: a comparative characterisation study, *Green Chemistry* 18 (2016) 2651–2665. <https://doi.org/10.1039/C5GC03043A>.
- [322] M. Ago, B.L. Tardy, L. Wang, J. Guo, A. Khakalo, O.J. Rojas, Supramolecular assemblies of lignin into nano- and microparticles, *MRS Bull* 42 (2017) 371–378. <https://doi.org/10.1557/MRS.2017.88/FIGURES/5>.
- [323] A. Eraghi Kazzaz, P. Fatehi, Fabrication of amphoteric lignin and its hydrophilicity/oleophilicity at oil/water interface, *J Colloid Interface Sci* 561 (2020) 231–243. <https://doi.org/10.1016/J.JCIS.2019.11.111>.
- [324] Ł. Klapiszewski, M. Nowacka, G. Milczarek, T. Jesionowski, Physicochemical and electrokinetic properties of silica/lignin biocomposites, *Carbohydr Polym* 94 (2013) 345–355. <https://doi.org/10.1016/J.CARBPOL.2013.01.058>.
- [325] Z. Wang, N. Bo, Y. Liu, G. Yang, Y. Liu, Y. Zhao, Lignin anion exchangers, *Bioresources* 8 (2013) 3505–3517.
- [326] I.A. Gilca, V.I. Popa, C. Crestini, Obtaining lignin nanoparticles by sonication, *Ultrason Sonochem* 23 (2015) 369–375. <https://doi.org/10.1016/J.ULTSONCH.2014.08.021>.
- [327] T. Worarutariyachai, S. Chuangchote, Carbon fibers derived from pure alkali lignin fibers through electrospinning with carbonization, *Bioresources* 15 (2020). <https://doi.org/10.15376/biores.15.2.2412-2427>.

- [328] M. Kumar, M. Hietala, K. Oksman, Lignin-based electrospun carbon nanofibers, *Front Mater* 6 (2019). <https://doi.org/10.3389/fmats.2019.00062>.
- [329] M.B. Stie, K. Kalouta, V. Vetri, V. Foderà, Protein materials as sustainable non- and minimally invasive strategies for biomedical applications, *Journal of Controlled Release* 344 (2022) 12–25. <https://doi.org/10.1016/j.jconrel.2022.02.016>.
- [330] K. Kalouta, M.B. Stie, X. Sun, V. Foderà, V. Vetri, Eco-Friendly Electrospun Nanofibers Based on Plant Proteins as Tunable and Sustainable Biomaterials, *ACS Sustain Chem Eng* 12 (2024) 10118–10129. <https://doi.org/10.1021/acssuschemeng.4c00895>.
- [331] I.L.R. Cintra, M.C. Rezende, L.M. Guerrini, L.R. Nahra, R.R. Lucas, L.S. Montagna, E.C. Botelho, Electrospinning of PAN/lignin blends aiming the production of carbon nanofibers, *MRS Commun* 14 (2024) 82–89. <https://doi.org/10.1557/S43579-023-00504-5/FIGURES/3>.
- [332] I. Korbog, S. Mohamed Saleh, Studies on the formation of intermolecular interactions and structural characterization of polyvinyl alcohol/lignin film, *International Journal of Environmental Studies* 73 (2016) 226–235. <https://doi.org/10.1080/00207233.2016.1143700>.
- [333] U. Riaz, S.M. Ashraf, Characterization of Polymer Blends with FTIR Spectroscopy, *Wiley Blackwell* 6 9783527331536 (2015) 625–678. <https://doi.org/10.1002/9783527645602.CH20>.
- [334] M. Ago, K. Okajima, J.E. Jakes, S. Park, O.J. Rojas, Lignin-based electrospun nanofibers reinforced with cellulose nanocrystals, *Biomacromolecules* 13 (2012). <https://doi.org/10.1021/bm201828g>.
- [335] M. Brogly, S. Bistac, D. Bindel, Advanced surface FTIR spectroscopy analysis of poly(ethylene)-block-poly(ethylene oxide) thin film adsorbed on gold substrate, *Appl Surf Sci* 603 (2022). <https://doi.org/10.1016/j.apsusc.2022.154428>.
- [336] C. Bergeron, E. Perrier, A. Potier, G. Delmas, A Study of the Deformation, Network, and Aging of Polyethylene Oxide Films by Infrared Spectroscopy and Calorimetric Measurements, *Int J Spectrosc* 2012 (2012) 1–13. <https://doi.org/10.1155/2012/432046>.
- [337] L.H. Sim, S.N. Gan, C.H. Chan, R. Yahya, ATR-FTIR studies on ion interaction of lithium perchlorate in polyacrylate/poly(ethylene oxide) blends, *Spectrochim Acta A Mol Biomol Spectrosc* 76 (2010). <https://doi.org/10.1016/j.saa.2009.09.031>.
- [338] K. Chrissopoulou, K.S. Andrikopoulos, S. Fotiadou, S. Bollas, C. Karageorgaki, D. Christofilos, G.A. Voyiatzis, S.H. Anastasiadis, Crystallinity and chain conformation in PEO/layered silicate nanocomposites, *Macromolecules* 44 (2011) 9710–9722. https://doi.org/10.1021/MA201711R/ASSET/IMAGES/LARGE/MA-2011-01711R_0012.JPEG.
- [339] X.X. Jiang, E. Naoko, Z.P. Zhong, Structure properties of pyrolytic lignin extracted from aged bio-oil, *Chinese Science Bulletin* 56 (2011). <https://doi.org/10.1007/s11434-011-4465-4>.
- [340] A. Maceda, T. Terrazas, Fluorescence Microscopy Methods for the Analysis and Characterization of Lignin, *Polymers (Basel)* 14 (2022). <https://doi.org/10.3390/polym14050961>.
- [341] Y. Xue, X. Qiu, X. Ouyang, Insights into the effect of aggregation on lignin fluorescence and its application for microstructure analysis, *Int J Biol Macromol* 154 (2020). <https://doi.org/10.1016/j.ijbiomac.2020.03.056>.
- [342] B. Chabbert, C. Terryn, M. Herbaut, A. Vaidya, A. Habrant, G. Paës, L. Donaldson, Fluorescence techniques can reveal cell wall organization and predict saccharification in pretreated wood biomass, *Ind Crops Prod* 123 (2018). <https://doi.org/10.1016/j.indcrop.2018.06.058>.
- [343] L.A. Donaldson, K. Radotic, Fluorescence lifetime imaging of lignin autofluorescence in normal and compression wood, *J Microsc* 251 (2013). <https://doi.org/10.1111/jmi.12059>.
- [344] Y. Lu, P. Jabbari, A. Mukhamedshin, A. V. Zvyagin, Fluorescence lifetime imaging in drug delivery research, *Adv Drug Deliv Rev* (2025) 115521. <https://doi.org/10.1016/J.ADDR.2025.115521>.
- [345] Y. Polat, E. Stojanovska, T.A. Negawo, E. Doner, A. Kilic, Lignin as an additive for advanced composites, *Green Energy and Technology* 0 (2017). https://doi.org/10.1007/978-3-319-46610-1_4.
- [346] M.R. Ridho, E.A. Agustiany, M. Rahmi Dn, E.W. Madyaratri, M. Ghozali, W.K. Restu, F. Falah, M.A. Rahandi Lubis, F.A. Syamani, Y. Nurhamiyah, S. Hidayati, A. Sohail, P. Karungamye, D.S. Nawawi, A.H. Iswanto, N.

- Othman, N.A. Mohamad Aini, M.H. Hussin, K. Sahakaro, N. Hayeemasae, M.Q. Ali, W. Fatriasari, Lignin as Green Filler in Polymer Composites: Development Methods, Characteristics, and Potential Applications, *Advances in Materials Science and Engineering* 2022 (2022). <https://doi.org/10.1155/2022/1363481>.
- [347] L. Guo, Y. Xu, Q. Xu, L. Jin, Nanocellulose-lignin films incorporating AgNPs for potential wound dressing: green synthesis, antioxidant ability, antibacterial capability and biocompatibility, *Cellulose* 31 (2024) 7569–7585. <https://doi.org/10.1007/S10570-024-06040-2/FIGURES/11>.
- [348] L. Guo, Y. Xu, Q. Xu, L. Jin, Nanocellulose-lignin films incorporating AgNPs for potential wound dressing: green synthesis, antioxidant ability, antibacterial capability and biocompatibility, *Cellulose* 31 (2024) 7569–7585. <https://doi.org/10.1007/S10570-024-06040-2/FIGURES/11>.
- [349] P. Terzioğlu, F.N. Parin, Y. Sıcak, Lignin Composites for Biomedical Applications: Status, Challenges and Perspectives, in: 2020. https://doi.org/10.1007/978-3-030-40663-9_9.
- [350] H. Faustino, N. Gil, C. Baptista, A.P. Duarte, Antioxidant Activity of Lignin Phenolic Compounds Extracted from Kraft and Sulphite Black Liquors, *Molecules* 2010, Vol. 15, Pages 9308–9322 15 (2010) 9308–9322. <https://doi.org/10.3390/MOLECULES15129308>.
- [351] D. Kai, W. Ren, L. Tian, P.L. Chee, Y. Liu, S. Ramakrishna, X.J. Loh, Engineering Poly(lactide)-Lignin Nanofibers with Antioxidant Activity for Biomedical Application, *ACS Sustain Chem Eng* 4 (2016) 5268–5276. https://doi.org/10.1021/ACSSUSCHEMENG.6B00478/ASSET/IMAGES/LARGE/SC-2016-00478G_0004.JPEG.
- [352] T. Dizhbite, G. Telysheva, V. Jurkane, U. Viesturs, Characterization of the radical scavenging activity of lignins - Natural antioxidants, *Bioresour Technol* 95 (2004). <https://doi.org/10.1016/j.biortech.2004.02.024>.
- [353] H. jia Xiang, A. ling Zhong, H. Wang, L. Xiao, T. ren Deng, T. gen Hu, P. Wen, Fabrication of alkali lignin-based emulsion electrospun nanofibers for the nanoencapsulation of beta-carotene and the enhanced antioxidant property, *Int J Biol Macromol* 218 (2022) 739–750. <https://doi.org/10.1016/J.IJBIOMAC.2022.07.121>.
- [354] A.G. Morena, T. Tzanov, Antibacterial lignin-based nanoparticles and their use in composite materials, *Nanoscale Adv* 4 (2022) 4447–4469. <https://doi.org/10.1039/D2NA00423B>.
- [355] Z. Xu, B. Liang, J. Tian, J. Wu, Anti-inflammation biomaterial platforms for chronic wound healing, *Biomater Sci* 9 (2021) 4388–4409. <https://doi.org/10.1039/D1BM00637A>.
- [356] G. Wang, F. Yang, W. Zhou, N. Xiao, M. Luo, Z. Tang, The initiation of oxidative stress and therapeutic strategies in wound healing, *Biomedicine & Pharmacotherapy* 157 (2023) 114004. <https://doi.org/10.1016/J.BIOPHA.2022.114004>.
- [357] J. Xu, J.J. Xu, Q. Lin, L. Jiang, D. Zhang, Z. Li, B. Ma, C. Zhang, L. Li, D. Kai, H.D. Yu, X.J. Loh, Lignin-Incorporated Nanogel Serving As an Antioxidant Biomaterial for Wound Healing, *ACS Appl Bio Mater* 4 (2021) 3–13. https://doi.org/10.1021/ACSABM.0C00858/ASSET/IMAGES/LARGE/MT0C00858_0007.JPEG.
- [358] L. Montanaro, C.R. Arciola, E. Cenni, G. Ciapetti, F. Savioli, F. Filippini, L.A. Barsanti, Cytotoxicity, blood compatibility and antimicrobial activity of two cyanoacrylate glues for surgical use, *Biomaterials* 22 (2000) 59–66. [https://doi.org/10.1016/S0142-9612\(00\)00163-0](https://doi.org/10.1016/S0142-9612(00)00163-0).
- [359] S. Thomas, A.R. Ajitha, C.J. Chirayil, B. Thomas, *Handbook of Biopolymers*, 2023. <https://doi.org/10.1007/978-981-19-0710-4>.
- [360] R. Xu, Y. Fang, Z. Zhang, Y. Cao, Y. Yan, L. Gan, J. Xu, G. Zhou, Recent Advances in Biodegradable and Biocompatible Synthetic Polymers Used in Skin Wound Healing, *Materials* 2023, Vol. 16, Page 5459 16 (2023) 5459. <https://doi.org/10.3390/MA16155459>.
- [361] A. Celebioglu, T. Uyar, Fast-dissolving antioxidant curcumin/cyclodextrin inclusion complex electrospun nanofibrous webs, *Food Chem* 317 (2020). <https://doi.org/10.1016/j.foodchem.2020.126397>.
- [362] U.E. Illangakoon, H. Gill, G.C. Shearman, M. Parhizkar, S. Mahalingam, N.P. Chatterton, G.R. Williams, Fast dissolving paracetamol/caffeine nanofibers prepared by electrospinning, *Int J Pharm* 477 (2014). <https://doi.org/10.1016/j.ijpharm.2014.10.036>.

- [363] Z. Yu Qin, X.W. Jia, Q. Liu, B. Hua Kong, H. Wang, Fast dissolving oral films for drug delivery prepared from chitosan/pullulan electrospinning nanofibers, *Int J Biol Macromol* 137 (2019). <https://doi.org/10.1016/j.ijbiomac.2019.06.224>.
- [364] X. Li, M.A. Kanjwal, L. Lin, I.S. Chronakis, Electrospun polyvinyl-alcohol nanofibers as oral fast-dissolving delivery system of caffeine and riboflavin, *Colloids Surf B Biointerfaces* 103 (2013). <https://doi.org/10.1016/j.colsurfb.2012.10.016>.
- [365] S. Zaidi, S.L. Singh, A.U. Khan, Exploring antibiofilm potential of bacitracin against streptococcus mutans, *Microb Pathog* 149 (2020). <https://doi.org/10.1016/j.micpath.2020.104279>.
- [366] R. Gul, M. Mir, M.N. Ali, An Appraisal of pH triggered Bacitracin drug release, through composite hydrogel systems, *J Biomater Appl* 37 (2023) 1699–1715. <https://doi.org/10.1177/08853282231160212>.
- [367] J.S. Glasser, C.H. Guymon, K. Mende, S.E. Wolf, D.R. Hospenenthal, C.K. Murray, Activity of topical antimicrobial agents against multidrug-resistant bacteria recovered from burn patients, *Burns* 36 (2010) 1172–1184. <https://doi.org/10.1016/j.burns.2010.05.013>.
- [368] S. Maitz, W. Schlemmer, M.A. Hobisch, J. Hobisch, M. Kienberger, Preparation and Characterization of a Water-Soluble Kraft Lignin, *Adv Sustain Syst* 4 (2020) 2000052. <https://doi.org/10.1002/ADSU.202000052>.
- [369] DPPH, antioxidant capacity | LIBIOS, (n.d.). <https://libios.fr/en/analytical-solutions/oxydative-stress-antioxidant-capacity/oxydative-stress-antioxidant-capacity-assay-kits/dpph-antioxidant-capacity> (accessed February 5, 2025).
- [370] N.B. Sadeer, D. Montesano, S. Albrizio, G. Zengin, M.F. Mahomoodally, The versatility of antioxidant assays in food science and safety—chemistry, applications, strengths, and limitations, *Antioxidants* 9 (2020) 1–39. <https://doi.org/10.3390/ANTIOX9080709>.
- [371] J. Petiti, L. Revel, C. Divieto, Standard Operating Procedure to Optimize Resazurin-Based Viability Assays, *Biosensors (Basel)* 14 (2024) 156. <https://doi.org/10.3390/BIOS14040156/S1>.
- [372] F. Bonnier, M.E. Keating, T.P. Wróbel, K. Majzner, M. Baranska, A. Garcia-Munoz, A. Blanco, H.J. Byrne, Cell viability assessment using the Alamar blue assay: A comparison of 2D and 3D cell culture models, *Toxicology in Vitro* 29 (2015) 124–131. <https://doi.org/10.1016/J.TIV.2014.09.014>.
- [373] G. Puleo, F. Terracina, V. Catania, S. Sciré, D. Schillaci, M. Licciardi, Electrosprayed Poly-butyl-succinate microparticles for sustained release of Ciprofloxacin as an antimicrobial delivery system, *Powder Technol* 432 (2024). <https://doi.org/10.1016/j.powtec.2023.119152>.
- [374] H. Lee, S. An, S. Kim, B. Jeon, M. Kim, & Ick, S. Kim, Readily Functionalizable and Stabilizable Polymeric Particles with Controlled Size and Morphology by Electrospray OPEN, *ScIENTIFIC REPORTS* | 8 (2018) 15725. <https://doi.org/10.1038/s41598-018-34124-0>.
- [375] C. Vilos, L.A. Velasquez, Therapeutic strategies based on polymeric microparticles, *J Biomed Biotechnol* 2012 (2012). <https://doi.org/10.1155/2012/672760>.
- [376] E. Lagreca, V. Onesto, C. Di Natale, S. La Manna, P.A. Netti, R. Vecchione, Recent advances in the formulation of PLGA microparticles for controlled drug delivery, *Progress in Biomaterials* 2020 9:4 9 (2020) 153–174. <https://doi.org/10.1007/S40204-020-00139-Y>.
- [377] W. Liu, C. Selomulya, X.D. Chen, Design of polymeric microparticles for pH-responsive and time-sustained drug release, *Biochem Eng J* 81 (2013) 177–186. <https://doi.org/10.1016/J.BEJ.2013.10.018>.
- [378] S. Freiberg, X.X. Zhu, Polymer microspheres for controlled drug release, *Int J Pharm* 282 (2004) 1–18. <https://doi.org/10.1016/J.IJPHARM.2004.04.013>.
- [379] A. Mtibe, S. Muniyasamy, T.C. Mokhena, O. Ofosu, V. Ojijo, M. John, Recent insight into the biomedical applications of polybutylene succinate and polybutylene succinate-based materials, *Express Polym Lett* 17 (2023) 2–28. <https://doi.org/10.3144/expresspolymlett.2023.2>.
- [380] M. Gigli, M. Fabbri, N. Lotti, R. Gamberini, B. Rimini, A. Munari, Poly(butylene succinate)-based polyesters for biomedical applications: A review in memory of our beloved colleague and friend Dr. Lara Finelli., *Eur Polym J* 75 (2016) 431–460. <https://doi.org/10.1016/j.eurpolymj.2016.01.016>.

Bibliography

- [381] L. Cicero, M. Licciardi, R. Cirincione, R. Puleio, G. Giammona, G. Giglia, P. Sardo, G. Edoardo Vigni, A. Cioffi, A. Sanfilippo, G. Cassata, Polybutylene succinate artificial scaffold for peripheral nerve regeneration, *J Biomed Mater Res B Appl Biomater* 110 (2022). <https://doi.org/10.1002/jbm.b.34896>.
- [382] G.E. Vigni, G. Cassata, G. Caldarella, R. Cirincione, M. Licciardi, G.C. Miceli, R. Puleio, L. D'Itri, R. Lo Coco, L. Camarda, L. Cicero, Improved Bone Regeneration Using Biodegradable Polybutylene Succinate Artificial Scaffold in a Rabbit Model, *J Funct Biomater* 14 (2023). <https://doi.org/10.3390/jfb14010022>.
- [383] G.C. Miceli, F.S. Palumbo, F.P. Bonomo, M. Zingales, M. Licciardi, Polybutylene Succinate Processing and Evaluation as a Micro Fibrous Graft for Tissue Engineering Applications, *Polymers (Basel)* 14 (2022). <https://doi.org/10.3390/polym14214486>.
- [384] G. Di Prima, M. Licciardi, F. Carfi Pavia, A.I. Lo Monte, G. Cavallaro, G. Giammona, Microfibrillar polymeric ocular inserts for triamcinolone acetonide delivery, *Int J Pharm* 567 (2019). <https://doi.org/10.1016/j.ijpharm.2019.118459>.
- [385] G. Gao, Y.W. Jiang, H.R. Jia, F.G. Wu, Near-infrared light-controllable on-demand antibiotics release using thermo-sensitive hydrogel-based drug reservoir for combating bacterial infection, *Biomaterials* 188 (2019). <https://doi.org/10.1016/j.biomaterials.2018.09.045>.
- [386] R. Davis, A. Markham, J.A. Balfour, Ciprofloxacin: An Updated Review of its Pharmacology, Therapeutic Efficacy and Tolerability, *Drugs* 51 (1996). <https://doi.org/10.2165/00003495-199651060-00010>.
- [387] S. Federico, G. Pitarresi, F.S. Palumbo, C. Fiorica, V. Catania, D. Schillaci, G. Giammona, An asymmetric electrospun membrane for the controlled release of ciprofloxacin and FGF-2: Evaluation of antimicrobial and chemoattractant properties, *Materials Science and Engineering C* 123 (2021). <https://doi.org/10.1016/j.msec.2021.112001>.
- [388] M. Jangde, S.K. Chatterjee, S. Ghosh, D. Sinha, R. Jangde, Ciprofloxacin Hydrochloride Mediated Enhanced Solubilization and Stability by UV-Spectroscopy, *J Pharm Res Int* (2022). <https://doi.org/10.9734/jpri/2022/v34i11b35535>.
- [389] L.É. Uhljar, S.Y. Kan, N. Radacsi, V. Koutsos, P. Szabó-Révész, R. Ambrus, In Vitro Drug Release, Permeability, and Structural Test of Ciprofloxacin-Loaded Nanofibers, *Pharmaceutics* 2021, Vol. 13, Page 556 13 (2021) 556. <https://doi.org/10.3390/PHARMACEUTICS13040556>.
- [390] S. Tripathi, B.N. Singh, D. Singh, G. kumar, P. Srivastava, Optimization and evaluation of ciprofloxacin-loaded collagen/chitosan scaffolds for skin tissue engineering, *3 Biotech* 11 (2021) 1–17. <https://doi.org/10.1007/S13205-020-02567-W/FIGURES/13>.
- [391] C. Günday, S. Anand, H.B. Gencer, S. Munafò, L. Moroni, A. Fusco, G. Donnarumma, C. Ricci, P.C. Hatir, N.G. Türeli, A.E. Türeli, C. Mota, S. Danti, Ciprofloxacin-loaded polymeric nanoparticles incorporated electrospun fibers for drug delivery in tissue engineering applications, *Drug Deliv Transl Res* 10 (2020) 706–720. <https://doi.org/10.1007/S13346-020-00736-1>.
- [392] S. Kanth, Y. Malgar Puttaiahgowda, S. Gupta, S. T, Recent advancements and perspective of ciprofloxacin-based antimicrobial polymers, *J Biomater Sci Polym Ed* 34 (2023) 918–949. <https://doi.org/10.1080/09205063.2022.2145872>.
- [393] D. Donà, E. Barbieri, G. Brigadoi, C. Liberati, S. Bosis, E. Castagnola, C. Colomba, L. Galli, L. Lancella, A. Lo Vecchio, M. Meschiari, C. Montagnani, M. De Luca, S. Mercadante, S. Esposito, State of the Art of Antimicrobial and Diagnostic Stewardship in Pediatric Setting, *Antibiotics* 2025, Vol. 14, Page 132 14 (2025) 132. <https://doi.org/10.3390/ANTIBIOTICS14020132>.
- [394] Y. Yang, N. Bajaj, P. Xu, K. Ohn, M.D. Tsifansky, Y. Yeo, Development of highly porous large PLGA microparticles for pulmonary drug delivery, *Biomaterials* 30 (2009) 1947–1953. <https://doi.org/10.1016/J.BIOMATERIALS.2008.12.044>.
- [395] H. Zhang, D. Edgar, P. Murray, A. Rak-Raszewska, L. Glennon-Alty, A.I. Cooper, Synthesis of Porous Microparticles with Aligned Porosity, *Adv Funct Mater* 18 (2008) 222–228. <https://doi.org/10.1002/ADFM.200701309>.
- [396] S.J. Gaskell, Electrospray: Principles and practice, *Journal of Mass Spectrometry* 32 (1997). [https://doi.org/10.1002/\(SICI\)1096-9888\(199707\)32:7<677::AID-JMS536>3.0.CO;2-G](https://doi.org/10.1002/(SICI)1096-9888(199707)32:7<677::AID-JMS536>3.0.CO;2-G).

- [397] M.E. Cam, Y. Zhang, M. Edirisinghe, Electrosprayed microparticles: a novel drug delivery method, *Expert Opin Drug Deliv* 16 (2019) 895–901. <https://doi.org/10.1080/17425247.2019.1648427>.
- [398] F. Siepmann, V. Le Brun, J. Siepmann, Drugs acting as plasticizers in polymeric systems: A quantitative treatment, *Journal of Controlled Release* 115 (2006) 298–306. <https://doi.org/10.1016/j.jconrel.2006.08.016>.
- [399] K. Mohanraj, S. Sethuraman, U.M. Krishnan, Development of poly(butylene succinate) microspheres for delivery of levodopa in the treatment of Parkinson's disease, *J Biomed Mater Res B Appl Biomater* 101 B (2013) 840–847. <https://doi.org/10.1002/JBM.B.32888>.
- [400] M.S. Refat, W.F. El-Hawary, M.A.A. Moussa, IR, ¹H NMR, mass, XRD and TGA/DTA investigations on the ciprofloxacin/iodine charge-transfer complex, *Spectrochim Acta A Mol Biomol Spectrosc* 78 (2011) 1356–1363. <https://doi.org/10.1016/J.SAA.2011.01.010>.
- [401] V. Ciaramitaro, E. Piacenza, P. Lo Meo, C. Librici, M.M. Calvino, P. Conte, G. Lazzara, D.F. Chillura Martino, From micro to macro: Physical-chemical characterization of wheat starch-based films modified with PEG200, sodium citrate, or citric acid, *Int J Biol Macromol* 253 (2023). <https://doi.org/10.1016/j.ijbiomac.2023.127225>.
- [402] H. Mesallati, A. Umerska, K.J. Paluch, L. Tajber, Amorphous Polymeric Drug Salts as Ionic Solid Dispersion Forms of Ciprofloxacin, *Mol Pharm* 14 (2017) 2209–2223. <https://doi.org/10.1021/acs.molpharmaceut.7b00039>.
- [403] F. Terracina, R. Caruana, F.P. Bonomo, F. Montalbano, M. Licciardi, Gastro-Resistant Microparticles Produced by Spray-Drying as Controlled Release Systems for Liposoluble Vitamins, *Pharmaceutics* 14 (2022). <https://doi.org/10.3390/PHARMACEUTICS14071480>.
- [404] A. Chua, H.T. Ng, W.S. Cheow, K. Hadinoto, Evaluating spray gelation and spray freeze drying as the granulation method to prepare oral tablets of amorphous drug nanoplex, *Advanced Powder Technology* 34 (2023). <https://doi.org/10.1016/j.apt.2023.104151>.
- [405] F. Tewes, J. Brillault, B. Lamy, P. Oconnell, J.C. Olivier, W. Couet, A.M. Healy, Ciprofloxacin-Loaded Inorganic-Organic Composite Microparticles to Treat Bacterial Lung Infection, *Mol Pharm* 13 (2016) 100–112. https://doi.org/10.1021/ACS.MOLPHARMACEUT.5B00543/SUPPL_FILE/MP5B00543_SI_001.PDF.
- [406] V. Volpe, M.N. Giacomodonato, D.O. Sordelli, M. Insausti, F.R. Buzzola, M. Grünhut, Ciprofloxacin loaded o/w microemulsion against *Staphylococcus aureus*. Analytical and biological studies for topical and intranasal administration, *J Drug Deliv Sci Technol* 57 (2020) 101705. <https://doi.org/10.1016/J.JDDST.2020.101705>.
- [407] A. Ducrotté-Tassel, P. Kirilov, J.P. Salvi, V.H. Tran, V. Dore, G. Marignac, C.P. Pignon, S. Perrot, R. Boulieu, Ex-vivo permeation of enrofloxacin through shed skin of *Python molurus bivittatus*, as evaluated with a Franz cell, *J Drug Deliv Sci Technol* 36 (2016) 89–94. <https://doi.org/10.1016/J.JDDST.2016.06.017>.
- [408] L.K. Vora, A.J. Courtenay, I.A. Tekko, E. Larrañeta, R.F. Donnelly, Pullulan-based dissolving microneedle arrays for enhanced transdermal delivery of small and large biomolecules, *Int J Biol Macromol* 146 (2020). <https://doi.org/10.1016/j.ijbiomac.2019.12.184>.
- [409] J. Ziesmer, P. Tajpara, N.J. Hempel, M. Ehrström, K. Melican, L. Eidsmo, G.A. Sotiriou, Vancomycin-Loaded Microneedle Arrays against Methicillin-Resistant *Staphylococcus Aureus* Skin Infections, *Adv Mater Technol* 6 (2021). <https://doi.org/10.1002/admt.202001307>.
- [410] M.A. Girasolo, D. Schillaci, C. Di Salvo, G. Barone, A. Silvestri, G. Ruisi, Synthesis, spectroscopic characterization and in vitro antimicrobial activity of diorganotin(IV) dichloride adducts with [1,2,4]triazolo-[1,5-a]pyrimidine and 5,7-dimethyl-[1,2,4]triazolo-[1,5-a]pyrimidine, *J Organomet Chem* 691 (2006) 693–701. <https://doi.org/10.1016/j.jorgchem.2005.10.007>.
- [411] A. Carbone, S. Cascioferro, B. Parrino, D. Carbone, C. Pecoraro, D. Schillaci, M.G. Cusimano, G. Cirrincione, P. Diana, Thiazole analogues of the marine alkaloid nortopsentin as inhibitors of bacterial biofilm formation, *Molecules* 26 (2021). <https://doi.org/10.3390/molecules26010081>.
- [412] J.N. O'Sullivan, M.C. Rea, P.M. O'Connor, C. Hill, R.P. Ross, Human skin microbiota is a rich source of bacteriocin-producing staphylococci that kill human pathogens, *FEMS Microbiol Ecol* 95 (2019). <https://doi.org/10.1093/femsec/fiy241>.
- [413] S.M. Lee, H.L. Keum, W.J. Sul, Bacterial Crosstalk via Antimicrobial Peptides on the Human Skin: Therapeutics from a Sustainable Perspective, *Journal of Microbiology* 61 (2023). <https://doi.org/10.1007/s12275-022-00002-8>.

- [414] S.H. Mohamed, M.S.M. Mohamed, M.S. Khalil, M. Azmy, M.I. Mabrouk, Combination of essential oil and ciprofloxacin to inhibit/eradicate biofilms in multidrug-resistant *Klebsiella pneumoniae*, *J Appl Microbiol* 125 (2018) 84–95. <https://doi.org/10.1111/JAM.13755>.
- [415] Y. Liu, J.H. Moore, G.L. Kolling, J.S. McGrath, J.A. Papin, N.S. Swami, Minimum bactericidal concentration of ciprofloxacin to *Pseudomonas aeruginosa* determined rapidly based on pyocyanin secretion, *Sens Actuators B Chem* 312 (2020) 127936. <https://doi.org/10.1016/J.SNB.2020.127936>.
- [416] F. Tewes, J. Brillault, B. Lamy, P. Oconnell, J.C. Olivier, W. Couet, A.M. Healy, Ciprofloxacin-Loaded Inorganic-Organic Composite Microparticles to Treat Bacterial Lung Infection, *Mol Pharm* 13 (2016) 100–112. <https://doi.org/10.1021/acs.molpharmaceut.5b00543>.
- [417] P.Ø. Jensen, A. Briaies, R.P. Brochmann, H. Wang, K.N. Kragh, M. Kolpen, C. Hempel, T. Bjarnsholt, N. Høiby, O. Ciofu, Formation of hydroxyl radicals contributes to the bactericidal activity of ciprofloxacin against *Pseudomonas aeruginosa* biofilms, (2013). <https://doi.org/10.1111/2049-632X.12120>.
- [418] A.P. Magalhães, S.P. Lopes, M.O. Pereira, Insights into cystic fibrosis polymicrobial consortia: The role of species interactions in biofilm development, phenotype, and response to in-use antibiotics, *Front Microbiol* 7 (2017) 234137. <https://doi.org/10.3389/FMICB.2016.02146/BIBTEX>.
- [419] D. Twilley, O. Reva, D. Meyer, N. Lall, Mupirocin Promotes Wound Healing by Stimulating Growth Factor Production and Proliferation of Human Keratinocytes, *Front Pharmacol* 13 (2022). <https://doi.org/10.3389/fphar.2022.862112>.
- [420] C.M. Wallenwein, V. Weigel, G. Hofhaus, N. Dhakal, W. Schatton, S. Gelperina, F.K. Groeber-Becker, J. Dressman, M.G. Wacker, Pharmaceutical Development of Nanostructured Vesicular Hydrogel Formulations of Rifampicin for Wound Healing, *Int J Mol Sci* 23 (2022). <https://doi.org/10.3390/ijms232416207>.
- [421] A.M. Juncan, D.G. Moisă, A. Santini, C. Morgovan, L.L. Rus, A.L. Vonica-țincu, F. Loghin, Advantages of Hyaluronic Acid and Its Combination with Other Bioactive Ingredients in Cosmeceuticals, *Molecules* 2021, Vol. 26, Page 4429 26 (2021) 4429. <https://doi.org/10.3390/MOLECULES26154429>.
- [422] C. Bucolo, G. Maugeri, S. Giunta, V. D'Agata, F. Drago, G.L. Romano, Corneal wound healing and nerve regeneration by novel ophthalmic formulations based on cross-linked sodium hyaluronate, taurine, vitamin B6, and vitamin B12, *Front Pharmacol* 14 (2023) 1109291. <https://doi.org/10.3389/FPHAR.2023.1109291/BIBTEX>.
- [423] J.M. Park, J.E. Choi, Y.K. Lee, J.K. Kim, Antioxidant and Anti-inflammatory Responses of Pycnogenol, Hibiscus Flower Extract, and Niacin in Skin Epithelial Cells, *Asia-Pacific Journal of Convergent Research Interchange* 9 (2023). <https://doi.org/10.47116/apjcri.2023.05.51>.