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USE OF COLLAGEN-COATED POLYCAPROLACTONE NANOFIBERS TO IMPROVE HEALING OF DIFFICULT WOUNDS

Settore scientifico-disciplinare: **MED-19**

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Summary

1. INTRODUCTION

1.1. Background and clinical relevance

Wound healing is a complex, multifaceted process that involves the coordination of various cellular and molecular events. This process is essential for restoring the integrity and function of the skin and underlying tissues following injury. Despite advancements in medical treatments, chronic wounds, such as diabetic ulcers, pressure sores, and venous leg ulcers, remain a significant challenge in clinical practice. These wounds fail to progress through the normal stages of healing, leading to prolonged patient suffering, increased healthcare costs, and a higher risk of infection and amputation [1].

The development of effective wound dressings is a critical aspect of wound management. Traditional dressings, such as gauze and bandages, primarily provide protection and absorb exudate but do not actively promote healing. In recent years, there has been a shift towards bioactive dressings that can modulate the wound environment, promote tissue regeneration, and prevent infection. Among these, nanofiber-based dressings have gained attention due to their ability to mimic the extracellular matrix (ECM), offering a conducive environment for cell growth and tissue repair [2].

Collagen, a major component of the ECM, plays a crucial role in wound healing by providing structural support and influencing cell behavior. Its biocompatibility and ability to interact with various cell types make it an ideal candidate for inclusion in wound dressings [3].

Silver nanoparticles (AgNPs), known for their potent antimicrobial properties, could be incorporated into wound dressings to prevent infections, which are a common complication in chronic wounds [4].

Polycaprolactone (PCL) is a synthetic, biodegradable polymer that has been widely used in biomedical applications, including tissue engineering and drug delivery. PCL's mechanical properties, coupled with its ability to degrade in the body [5].

This PhD project explores the use of collagen and AgNPs coated PCL nanofiber membranes as a novel concept for wound dressings. The integration of these materials aims to combine the structural and biological benefits of collagen with the antimicrobial efficacy of AgNPs, all within the versatile framework of PCL nanofibers. This innovative approach has the potential to enhance wound healing outcomes by addressing multiple aspects of the healing process, including infection control, tissue regeneration, and support for cell migration and proliferation.

By investigating this new dressing concept, the project seeks to contribute to the ongoing development of advanced wound care solutions that can improve patient outcomes and reduce the burden of chronic wounds on healthcare systems.

1.2. Current challenges in wound healing

Wound healing, particularly in chronic and complex wounds, presents significant challenges that can impede the normal repair process and lead to prolonged healing times, increased risk of complications, and substantial healthcare costs. Several factors contribute to these challenges and understanding them is crucial for developing more effective therapeutic strategies [6].

1.2.1. Chronic wounds and delayed healing

Wound healing is a complex, dynamic biomechanical process through which the body attempts to restore the integrity of traumatized or devitalized tissues. Normally, this process involves a well-coordinated sequence of events, including hemostasis, inflammation, proliferation, and remodeling, leading to the closure and repair of the wound. However, when these stages are

disrupted or prolonged, the wound may become chronic, leading to significant challenges in management and treatment [7].

Chronic wounds are defined as longstanding tissue injuries that fail to progress through the normal stages of healing and do not respond to conventional methods of wound dressing or closure. This failure can be attributed to a range of factors, both local and systemic. Local factors include the depth of the wound, presence of infection, compromised vascular supply, and conditions like peripheral vascular disease or sustained pressure that impede healing. Systemic factors encompass metabolic disorders such as diabetes mellitus, immunodeficiencies, and nutritional deficiencies, all of which can severely impair the body's natural healing processes [6].

Recently, the term "complex wounds" has been introduced to more accurately describe these multifactorial, dynamic tissue healing challenges. For a wound to be classified as a complex wound, it must exhibit one or more of the following characteristics:

- persistence for more than three months;
- compromised vascularity or presence of necrotic tissue;
- presence of infection;
- associated comorbidities that impair the healing potential, such as diabetes or immunosuppression [8].

Complex wounds, due to their intricate nature, impose a significant burden on both individual patients and healthcare systems. On a personal level, they lead to a reduced quality of life, causing pain, discomfort, and limited mobility. On a broader institutional level, the economic cost is substantial. It is estimated that 1 to 3% of healthcare expenses in developed countries are dedicated to the management of complex wounds. In the United States alone, this represents

approximately $25 billion annually, a figure expected to rise as the prevalence of chronic conditions in the population increases [1,9,10].

The persistent nature of chronic wounds is often due to their tendency to remain in a prolonged inflammatory phase. During this phase, excessive production of inflammatory cytokines and proteases leads to the degradation of the ECM, preventing the formation of new tissue [11]. The chronic inflammatory environment is further exacerbated by factors such as compromised vascularity, hypoxia, and biofilm formation. Biofilms, which are communities of bacteria encased in a protective matrix, are particularly problematic because they render the wound more resistant to both the host immune response and antibiotic treatments, creating a cycle of ongoing infection and delayed healing [12].

In patients with conditions like diabetes, compromised blood flow reduces the supply of oxygen and essential nutrients to the wound site, contributing to the persistence of the wound. Venous insufficiency in patients with venous leg ulcers leads to increased venous pressure, edema, and further tissue damage, all of which hinder the wound healing process [13].

Given the multifactorial nature of chronic and complex wounds, their management requires a multidisciplinary approach that addresses both local and systemic factors. This complexity underscores the need for advanced wound care strategies that go beyond traditional methods. The development of innovative dressings that can modulate the wound environment, promote tissue regeneration, and manage infection is crucial for improving outcomes in patients with chronic wounds and reducing the overall burden on healthcare systems.

1.2.2. Infection risk

Infection is a major concern in wound management. Chronic wounds, due to their prolonged exposure and often compromised blood supply, are particularly susceptible to bacterial

colonization and subsequent infection. Infected wounds not only delay healing but also increase the risk of systemic infections, which can lead to more severe complications, including sepsis and amputation. The rise of antibiotic-resistant bacteria further exacerbates the challenge, necessitating the development of dressings with potent antimicrobial properties [14].

1.2.3. Need for bioactive dressings

To overcome the limitations of traditional dressings, there is a growing interest in bioactive dressings that can create a favorable microenvironment for wound healing. Such dressings are designed to modulate the wound environment actively, promoting cell proliferation, angiogenesis, and collagen deposition while also preventing microbial growth. However, the development of these advanced dressings is not without its challenges. Balancing the biocompatibility, mechanical properties, and degradation rates of the materials involved is essential to ensure that they support healing without causing adverse reactions [15].

1.2.4. Integration of multiple functions

One of the main challenges in developing next-generation wound dressings is integrating multiple functions into a single material. An ideal dressing should not only provide physical protection and manage exudate but also support tissue regeneration, offer antimicrobial protection, and degrade at a rate that matches the wound healing process. Achieving this level of multifunctionality requires the careful selection and combination of materials with complementary properties.

1.3. Objectives of the study

The primary objective of this PhD project is to explore the development and evaluation of a novel wound dressing concept that integrates collagen and AgNPs coated PCL nanofiber

membranes. This innovative approach aims to address the multifaceted challenges associated with chronic and complex wounds by leveraging the synergistic properties of these materials to enhance the wound healing process.

1.3.1. Development of nanofiber membranes

The first key objective in the development of nanofiber membranes is to establish and refine a fabrication process that integrates PCL nanofibers with collagen and AgNPs. This process aims to create a multifunctional membrane designed for effective wound healing, with a focus on enhancing biocompatibility, structural integrity, and antimicrobial properties.

To achieve this, electrospinning techniques are employed to produce PCL nanofibers. Electrospinning is a highly effective method that draws polymer solutions into ultra-fine fibers using an electrical field [16]. This allows for the formation of a porous, three-dimensional scaffold with nanoscale fiber diameters that closely resemble the architecture of the ECM [17]. The ECM-like structure is critical because it offers a conducive environment for cellular attachment, proliferation, and migration, which are essential processes in tissue regeneration and wound healing. The high surface area and porosity of the nanofibers further enhance their ability to facilitate gas exchange and maintain a moist wound environment, which is crucial for efficient healing [18].

Once the PCL nanofibers are fabricated, the next step involves coating them with collagen. Collagen is a natural protein that is abundant in human tissues and plays a vital role in wound healing by promoting cell adhesion and tissue regeneration. By applying a collagen coating to the PCL nanofibers, the biocompatibility of the membrane is significantly improved. This collagen layer helps mimic the natural healing environment of the body, supporting critical cellular activities such as cell signaling, proliferation, and migration. Moreover, collagen

contributes to the overall mechanical stability of the membrane, ensuring that it remains durable while still allowing for flexibility and conformability to the wound surface [19].

In addition to the collagen coating, AgNPs are incorporated into the nanofiber membrane. Silver has long been known for its antimicrobial properties, making it an ideal agent for preventing wound infections. By integrating AgNPs within the membrane, the material gains the ability to inhibit the growth of bacteria and other pathogens that could otherwise complicate the healing process. The presence of AgNPs ensures that the membrane maintains a sterile wound environment, reducing the risk of infection and supporting faster, more effective healing. Furthermore, the controlled release of silver ions from the nanoparticles allows for sustained antimicrobial activity over time, offering prolonged protection for chronic or slow-healing wounds [20].

The combination of PCL nanofibers, collagen coating, and AgNPs results in a versatile and advanced wound dressing that addresses multiple aspects of the healing process. The nanofiber structure provides a scaffold for cell growth and tissue regeneration, while the collagen coating enhances biocompatibility and supports cellular activities. Meanwhile, the AgNPs offer antimicrobial protection, ensuring that the wound remains free from infections, which is critical for optimal recovery. Through careful optimization of the fabrication process, these elements are brought together to create a novel dressing concept that has the potential to significantly improve wound care outcomes.

1.3.2. Characterization of membranes

The second objective focuses on the detailed characterization of the physicochemical properties of the nanofiber membranes developed for wound healing applications. Characterizing these properties is crucial for understanding how the membranes will perform in a clinical setting and ensuring they meet the requirements necessary for effective wound care.

A key aspect of this characterization process involves assessing the morphological characteristics of the membranes. This is typically achieved using advanced imaging techniques such as scanning electron microscopy (SEM) and atomic force microscopy (AFM). SEM provides high-resolution images that allow for a thorough examination of the surface structure of the nanofibers, including their diameter, porosity, and fiber distribution [21]. These parameters are critical in determining how well the membrane can mimic the ECM and support cellular activities essential for tissue regeneration. AFM, on the other hand, allows for a more in-depth analysis of the surface topology and mechanical properties of the fibers at the nanoscale, providing insight into the surface roughness, stiffness, and interaction of AgNPs and collagen with the nanofibers [22]. Understanding these morphological features is essential for optimizing the fabrication process and ensuring that the membranes exhibit the desired structural properties for wound healing.

In addition to morphological assessment, it is also vital to evaluate the mechanical properties of the nanofiber membranes. Wound dressings must be durable and flexible enough to withstand the mechanical stresses associated with movement and changes in the wound environment. Mechanical testing, which includes measuring tensile strength, elasticity, and flexibility, helps ensure that the membranes possess the necessary strength to remain intact during application while still being pliable enough to conform to irregular wound surfaces. These mechanical properties are particularly important when dealing with chronic wounds, where prolonged contact between the dressing and the wound is required. If the membranes are too rigid, they may cause discomfort or restrict natural movements, while insufficient strength could lead to premature degradation or tearing, compromising the healing process.

Through this comprehensive characterization process, it becomes possible to tailor the nanofiber membranes to specific wound healing needs. By understanding their structural, mechanical, and degradation characteristics, the membranes can be optimized to ensure they

provide effective and sustained support for tissue regeneration, while simultaneously maintaining a sterile and conducive environment for wound healing. This detailed analysis helps to bridge the gap between the laboratory development of the membranes and their practical application in clinical settings [23].

1.3.3. Biological evaluation in vitro

The third objective of this research is to carry out in vitro biological evaluations to assess the biocompatibility, cytotoxicity, and antimicrobial efficacy of the collagen and AgNPs coated PCL nanofiber membranes. These evaluations are critical for determining the potential of these membranes as a safe and effective wound care dressing, ensuring they perform their intended functions without adverse effects.

Biocompatibility testing is one of the first steps in the biological evaluation process. For the membranes to be effective in wound healing, they must support the growth and proliferation of cells essential to tissue repair. This includes cell types like fibroblasts, which are responsible for producing the ECM and collagen, and keratinocytes, which are involved in re-epithelialization and the restoration of the skin barrier. In vitro tests will involve initially culturing fibroblasts on the nanofiber membranes to assess how well they adhere, spread, and proliferate over time [24]. A membrane that promotes these cellular activities will be highly conducive to the healing process, facilitating faster tissue regeneration and closure of the wound. Furthermore, the biocompatibility of the membranes will provide insight into how well the collagen coating functions as a scaffold for cellular activity, mimicking the natural wound environment and encouraging tissue repair.

Alongside biocompatibility, cytotoxicity testing is also vital. Cytotoxicity refers to the potential of a material to cause damage to living cells, which in the case of wound dressings, could lead to delayed healing or additional complications. To confirm that the PCL nanofiber membranes

are safe for use in wound care, cytotoxicity assays will be conducted to evaluate whether the materials used, particularly the AgNPs and collagen, cause any harmful effects to the cells they come into contact with. This step is essential because, while AgNPs are known for their antimicrobial properties, they can be toxic at certain concentrations. Ensuring that the concentration and release rate of AgNPs are safe and do not harm cells is crucial for the clinical success of the membranes [25]. The results of these cytotoxicity tests will help optimize the balance between antimicrobial efficacy and safety, ensuring that the membranes promote healing without causing cytotoxic effects.

Another fundamental aspect of the biological evaluation is the assessment of antimicrobial activity. One of the primary benefits of incorporating AgNPs into the membranes is to prevent infections, a common complication in wound healing, especially in chronic or complex wounds [26]. In vitro antimicrobial tests will be performed to evaluate how effectively the membranes inhibit the growth of common wound pathogens, such as *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Enterococcus Faecium*. These tests will demonstrate whether the membranes can maintain a sterile environment by actively killing or inhibiting the growth of these microbes. The antimicrobial efficacy of the AgNPs will be observed over time to ensure that they provide sustained protection against infection, which is particularly important in wounds that are exposed to external contamination or where there is a high risk of infection due to compromised immune responses.

Through these biological evaluations, the safety and efficacy of the collagen and AgNPs coated PCL nanofiber membranes will be thoroughly assessed. A positive outcome from these tests will indicate that the membranes are not only biocompatible and non-toxic but also capable of preventing infections, making them a valuable tool for improving wound care. The combination of biocompatibility, cytotoxicity, and antimicrobial efficacy testing provides a comprehensive

understanding of how the membranes will function in a clinical setting, offering crucial data to support their potential use in advanced wound healing applications.

1.3.4. Clinical implications and future directions

The final objective of this research is to interpret the results obtained from the development and testing of the membranes in the context of their potential clinical applications. This stage focuses on assessing the feasibility of these membranes as an advanced wound dressing, particularly for the treatment of chronic and complex wounds.

One of the potential limitations of this research could be the need for further validation of the membrane's long-term performance in vivo. While in vitro tests provide valuable insights into biocompatibility, cytotoxicity, and antimicrobial properties, the complex environment of a living organism presents additional challenges. Variables such as the body’s immune response, varying wound conditions, and patient-specific factors may affect how the membranes perform in actual clinical scenarios. The research will, therefore, propose further in vivo studies to validate the safety, efficacy, and long-term stability of the membranes under different wound conditions. Additionally, optimizing the release rate of AgNPs and adjusting the mechanical properties of the membranes to suit different types of wounds may require further refinement. Identifying these gaps in the current research will help pave the way for additional studies aimed at improving the dressing’s design and performance.

Beyond the technical aspects of the study, this stage of the research will also reflect on the broader implications of using nanofiber membranes for wound care. Chronic wounds are a significant burden on healthcare systems worldwide, consuming substantial resources in terms of time, labor, and costs. The introduction of an effective, multifunctional dressing that can accelerate healing and prevent complications could have far-reaching effects on patient outcomes and healthcare economics. Faster healing times mean reduced hospital stays and

fewer follow-up visits, while a reduction in infections lowers the need for antibiotics and complex interventions [27]. This technology, therefore, holds the potential not only to improve the quality of life for patients suffering from chronic wounds but also to alleviate the economic burden associated with long-term wound care. The research will highlight these potential benefits, underscoring the value of continued investment in the development and commercialization of nanofiber-based dressings.

1.4. Wound healing processes

Wound healing is a dynamic and complex physiological process aimed at restoring the integrity and function of damaged tissues. It involves a series of well-coordinated events that can be broadly categorized into four overlapping phases: hemostasis, inflammation, proliferation, and remodeling [28]. Each phase is essential for proper wound closure and tissue repair. Understanding these phases provides a foundation for appreciating the challenges associated with chronic wounds and the potential benefits of advanced wound care technologies.

1.4.1. Hemostasis

The initial response to tissue injury is hemostasis, which begins immediately after the wound occurs. The primary goals of this phase are to stop bleeding and initiate the healing process. Hemostasis involves:

- **Vascular constriction:** the blood vessels constrict to reduce blood flow to the injured area, minimizing blood loss.
- **Platelet activation and aggregation:** platelets adhere to the exposed collagen and form a platelet plug, which is essential for clot formation [29].

- **Clot formation:** coagulation factors are activated, leading to the formation of a fibrin clot that stabilizes the wound and provides a matrix for incoming cells. This clot also releases growth factors and cytokines that signal the next phases of healing [30].

1.4.2. Inflammation

The inflammatory phase follows hemostasis and is characterized by the removal of debris, pathogens, and damaged tissue. This phase typically lasts for a few days and involves:

- **Vasodilation and increased permeability:** blood vessels dilate and become more permeable, allowing immune cells, such as neutrophils and macrophages, to migrate into the wound site.
- **Immune cell activity:** neutrophils are the first responders, phagocytosing bacteria and debris. They are later replaced by macrophages, which continue to clear debris and secrete cytokines that regulate inflammation and attract other cells necessary for healing [31]. During this phase, inflammatory cells release a variety of cytokines and growth factors, such as transforming growth factor-beta and platelet-derived growth factor [7].
- **Resolution of inflammation:** once the wound is cleaned, the inflammatory response subsides, and the focus shifts to tissue repair and regeneration. Chronic inflammation can occur if the wound is infected or if there are persistent irritants, which can delay healing.

1.4.3. Proliferation

The proliferation phase is characterized by the formation of new tissue to replace the damaged tissue. This phase typically begins a few days after injury and can last for several weeks. Key processes include:

- **Angiogenesis:** the formation of new blood vessels from pre-existing ones, which supplies oxygen and nutrients to the regenerating tissue [32].
- **Fibroplasia:** fibroblasts proliferate and produce collagen and ECM components, forming granulation tissue that fills the wound bed [33,34].
- **Re-epithelialization:** keratinocytes migrate across the wound bed, forming a new epithelial layer that covers the wound [35].
- **Wound contraction:** myofibroblasts at the wound edges contract, reducing the size of the wound and facilitating closure [36].

1.4.4. Remodeling

The remodeling phase is the final stage of wound healing and can last for several months to years. During this phase, the newly formed tissue is reorganized and strengthened:

- **Collagen remodeling:** type III collagen, initially deposited during the proliferation phase, is gradually replaced by type I collagen, which provides greater tensile strength [37].
- **Matrix maturation:** the ECM is remodeled to achieve its mature structure and composition, which supports tissue strength and function.
- **Scar formation:** the wound undergoes contraction and maturation, resulting in scar tissue that may differ in appearance and function from the original tissue. While scar tissue strengthens the wound, it may have different properties, such as reduced elasticity [38].

Understanding the phases of wound healing is crucial for developing effective treatments and technologies for wound management. Each phase plays a specific role in restoring tissue integrity and function, and disruptions at any stage can lead to chronic or delayed healing.

Advanced wound dressings and therapeutic approaches, such as the novel membranes explored in this project, aim to support and enhance these natural healing processes.

1.5. Collagen: role and clinical applications

Collagen is a primary structural protein in the ECM of connective tissues. It provides mechanical support, structural integrity, and contributes to cellular functions essential for tissue repair and regeneration. During the proliferation phase of wound healing, collagen is synthesized and deposited by fibroblasts, forming the granulation tissue that fills the wound bed [39].

1.5.1. Role of Collagen in wound healing

Collagen plays a crucial role in wound healing due to its involvement in several essential processes that contribute to tissue repair and regeneration. One of its primary functions is providing structural support during wound healing. Collagen fibers form a scaffold that acts as a framework for new tissue formation, helping to maintain the architecture and strength of the tissue throughout the healing process [40]. This structural support is vital, as it ensures that the wound maintains integrity while new cells and tissues are being developed.

In addition to providing structural support, collagen is instrumental in cell adhesion and migration, two key aspects of wound healing. Cells like fibroblasts, which produce the ECM, and keratinocytes, which are responsible for skin re-epithelialization, use collagen as a substrate for attachment. By adhering to collagen fibers, these cells are able to proliferate and migrate efficiently into the wound bed, promoting tissue regeneration. This cellular activity is critical for closing the wound and forming new tissue, making collagen an essential component in this phase of healing [41].

Collagen also plays a significant role in matrix remodeling, the final phase of wound healing, as previously reported. During this phase, the ECM is reorganized to strengthen the newly formed tissue. Initially, type III collagen is deposited in the wound during the early stages of healing. Over time, this is replaced by the more durable type I collagen, which contributes to the maturation and stabilization of the scar tissue. This replacement process is critical for ensuring that the repaired tissue has the necessary strength and resilience to withstand normal physiological stresses [37].

Additionally, collagen is involved in growth factor binding, which is essential for regulating various cellular processes. Collagen has the ability to bind and present growth factors, such as transforming growth factor-beta (TGF-β), to cells within the wound. These growth factors play a vital role in controlling cell proliferation, differentiation, and the synthesis of ECM components. By facilitating the availability of growth factors, collagen enhances the body's ability to orchestrate the complex biological events required for efficient and successful wound healing [42,43].

Through these multifaceted roles, collagen proves to be integral not only for providing structural support but also for driving cellular activities and remodeling processes that are essential for tissue repair.

1.5.2. Clinical applications of Collagen

Collagen's biocompatibility and functional properties make it highly valuable in a wide range of clinical applications, particularly in wound care and tissue engineering. One of its primary uses is in wound dressings. Collagen-based dressings are designed to create a favorable environment for wound healing by promoting tissue repair [3]. These dressings enhance cellular proliferation, improve the quality of the wound bed, and support the process of re-epithelialization, where new skin forms over the wound. Collagen dressings are especially

beneficial in treating chronic wounds, such as diabetic ulcers and venous leg ulcers, where traditional treatments often fail. By providing a structural matrix that supports new tissue growth, these dressings facilitate wound closure, helping to restore the integrity of damaged tissue.

Collagen is also used in dermal substitutes [44], particularly in reconstructive surgery and burn care. These substitutes act as temporary scaffolds that not only support the growth of new tissue but also integrate with the host tissue over time [45]. In the treatment of severe burns or traumatic injuries, collagen-based dermal substitutes are invaluable in covering large wound areas. By promoting tissue regeneration and healing, these substitutes help patients recover more effectively from extensive skin damage. Their ability to blend with the body's natural tissues makes them highly effective in supporting the complex process of wound healing in such cases [46].

In tissue engineering, collagen plays a crucial role as a material for scaffolds that mimic the ECM. These scaffolds provide the necessary support for cells to grow, organize, and form new tissues or organs. In this capacity, collagen-based scaffolds are used to engineer a variety of tissues, including skin, cartilage, and bone [47]. The resemblance of collagen to the body's natural ECM allows for better cellular interaction and tissue development. This application holds great promise for regenerative medicine, where collagen scaffolds could be used to repair or replace damaged tissues, ultimately improving patient outcomes in tissue regeneration and transplantation.

Through its use in wound dressings, dermal substitutes, and tissue engineering, collagen demonstrates its versatility and value in modern medicine. Its ability to integrate with human tissue, support cellular growth, and promote healing underscores its importance in both immediate clinical applications and innovative regenerative therapies.

1.5.3. Limitations and considerations

While collagen offers substantial benefits in wound healing and tissue engineering, there are notable limitations and considerations that must be addressed. One of the primary concerns is the source and purity of collagen. Collagen is commonly derived from animal sources, such as bovine or porcine tissue, which can raise issues related to purity and the potential for immune responses. Some individuals may react to animal-derived collagen, which could complicate healing or lead to rejection in certain applications. Moreover, there are ethical concerns associated with using animal-derived materials, which has spurred interest in developing recombinant collagen and synthetic alternatives. These alternatives aim to minimize immune reactions while addressing ethical considerations, making collagen-based treatments more universally acceptable and safer for a broader range of patients.

Another important factor to consider is the degradation rate of collagen-based materials. In wound healing and tissue regeneration, the degradation of collagen must be precisely controlled to align with the rate at which new tissue is formed. If the collagen degrades too quickly, it may not provide adequate structural support, potentially leading to insufficient tissue repair. Conversely, if the collagen degrades too slowly, it can hinder the integration of new tissue, delaying the healing process and potentially causing complications. The challenge is to create collagen-based materials with a degradation profile that is tailored to the specific needs of the wound or tissue being treated [48,49].

Cost is another significant limitation of collagen-based products. These materials, especially high-quality or recombinant collagen, can be expensive, which limits their accessibility, particularly in low-resource settings or for patients without adequate healthcare coverage. The high cost of production, purification, and clinical application poses a barrier to the widespread adoption of collagen-based wound care and tissue engineering solutions. Reducing the cost of

these products while maintaining their efficacy is a critical area for future research and development, with the goal of making these advanced treatments more accessible to a broader population.

1.6. Silver Nanoparticles: antimicrobial properties and applications in Wound Care

AgNPs have garnered significant attention in wound care due to their potent antimicrobial properties, making them valuable in the development of advanced wound dressings [50]. These nanoparticles combat a broad spectrum of pathogens, including bacteria, fungi, and viruses, through several key mechanisms. AgNPs disrupt bacterial cell membranes, increasing permeability and leading to cell lysis. They also generate reactive oxygen species (ROS) within microbial cells, causing oxidative stress that damages critical cellular components such as DNA, proteins, and lipids, ultimately resulting in cell death. Furthermore, AgNPs can inhibit enzymatic functions by binding to microbial enzymes, particularly those with thiol groups, thereby disrupting vital cellular processes like respiration and replication. Additionally, AgNPs can penetrate microbial cells and interact with nucleic acids, impairing DNA replication and transcription, which prevents microorganisms from reproducing and surviving [51]. These combined effects make AgNPs highly effective against a broad array of pathogens, including antibiotic-resistant strains such as Pseudomonas Aeruginosa and VRE Vancomycin Resistant Enterococcus.

In wound care, AgNPs are incorporated into various products to prevent infections and promote healing. Silver-impregnated dressings are among the most common applications, providing sustained antimicrobial activity at the wound site by continuously releasing silver ions that inhibit microbial growth [52]. These dressings are particularly effective in managing chronic wounds, burns, and surgical sites prone to infection. Additionally, AgNPs are formulated into

topical gels and creams for direct wound application, creating a protective barrier while delivering silver ions to reduce microbial load and support healing [20,53]. AgNPs are also often combined with other materials, such as collagen or polymeric scaffolds, to create multifunctional wound dressings that offer antimicrobial protection and support tissue regeneration and moisture balance, key factors in optimal wound healing [54].

The use of AgNPs in wound care offers several advantages. Their broad-spectrum antimicrobial activity makes them versatile in preventing and managing wound infections. Unlike traditional antibiotics, which target specific bacterial processes, AgNPs affect multiple cellular pathways, reducing the likelihood of resistance development. Moreover, by preventing infection, AgNPs help maintain a clean wound environment, conducive to faster and more effective healing. Research has also shown that AgNPs promote wound closure and reduce inflammation, further supporting the healing process [55,56].

However, the use of AgNPs is not without challenges. Concerns about toxicity arise at high concentrations, where AgNPs can be cytotoxic to human cells, potentially delaying wound healing or causing adverse reactions [25]. Therefore, controlling the concentration and release rate of AgNPs in wound dressings is crucial to minimize toxicity while maintaining antimicrobial efficacy. Additionally, the environmental impact of AgNPs, particularly their accumulation in water systems and potential effects on aquatic life, is a growing concern [57]. Ongoing research aims to develop sustainable and eco-friendly approaches to using AgNPs in wound care. Lastly, silver-based wound care products tend to be more expensive than traditional treatments, which may limit their accessibility, particularly in low-resource settings. The cost-effectiveness of AgNPs must be weighed against their long-term benefits in reducing infection rates and promoting healing.

1.7. Polycaprolactone (PCL): properties and applications in nanofiber membranes

PCL is a biodegradable polyester with a range of properties that make it an attractive material for biomedical applications, particularly in wound healing and tissue engineering.

PCL is known for its excellent biocompatibility, making it suitable for use in medical devices and tissue engineering scaffolds. Its biodegradability is another significant advantage, as PCL degrades slowly in biological environments through hydrolysis of its ester linkages. This slow degradation rate allows for sustained support of tissue regeneration over extended periods, making it ideal for applications where long-term structural integrity is required. Additionally, PCL is mechanically flexible, which is beneficial for applications that require conformability to irregular wound surfaces [58].

One of the most significant applications of PCL in wound care is in the fabrication of nanofiber membranes using electrospinning techniques. Electrospinning allows the production of ultra-thin PCL fibers with diameters ranging from nanometers to micrometers, creating a highly porous membrane with a large surface area. These nanofiber membranes mimic the structure of the ECM, providing a supportive environment for cell attachment, proliferation, and migration. The high porosity of PCL nanofiber membranes also facilitates the exchange of gases and fluids, which is crucial for maintaining a moist wound environment, an essential factor for efficient wound healing [59].

PCL nanofiber membranes have been explored for various applications in wound care due to their ability to be easily modified and combined with other bioactive materials. For instance, PCL can be blended with natural polymers like collagen or chitosan to enhance its biocompatibility and promote faster wound healing. Additionally, PCL nanofibers can be

coated with antimicrobial agents, such as AgNPs, to provide additional protection against infection. The versatility of PCL allows for the creation of multifunctional wound dressings that not only protect the wound from external contaminants but also actively promote tissue regeneration and healing [60-62].

Despite its many advantages, there are some challenges associated with the use of PCL in wound care. One of the primary concerns is its relatively slow degradation rate, which, while beneficial in some contexts, may not be ideal for all wound types. In cases where rapid degradation and resorption are desired, alternative materials or blends with faster-degrading polymers may be more appropriate. Additionally, PCL's hydrophobic nature can limit cell adhesion and proliferation, which has led to the development of surface modification techniques to improve its hydrophilicity and enhance its interaction with biological tissues [63].

1.8. Current wound dressings: advantages and limitations

Current wound dressings are essential in managing a wide variety of wounds, from acute injuries to chronic and complex wounds. These dressings provide protection, promote healing, and help maintain an optimal environment for tissue regeneration. However, while they offer several advantages, there are also limitations that impact their effectiveness and clinical outcomes [64].

One of the primary advantages of modern wound dressings is their ability to maintain a moist wound environment, which is critical for promoting cellular activities involved in healing, such as proliferation and migration [65]. Dressings such as hydrogels, hydrocolloids, and foams are specifically designed to keep the wound moist while absorbing excess exudate, preventing maceration of surrounding tissues. This moisture retention accelerates healing by facilitating autolytic debridement, a process where the body naturally removes dead tissue [66]. Additionally, many dressings are designed to be transpirant, allowing for gas exchange while

still protecting the wound from external contaminants. This feature is particularly beneficial in reducing infection risks.

Another advantage of current wound dressings is the availability of advanced materials that can be tailored to specific wound types. For instance, antimicrobial dressings containing agents like silver or iodine are used in wounds prone to infection, helping to reduce microbial load and prevent complications. Other dressings, such as alginates and collagen-based dressings, promote healing in chronic or deep wounds by providing structural support and enhancing cellular activities essential for tissue repair [64,67-69].

However, despite these benefits, there are notable limitations to current wound dressings. One significant limitation is that traditional dressings may not adequately address the multifactorial nature of chronic wounds. Chronic wounds, such as diabetic foot ulcers and venous leg ulcers, often result from complex underlying conditions like poor vascularization, infection, or systemic diseases, and conventional dressings alone may not be sufficient to promote complete healing. These wounds may require more advanced interventions, such as growth factor therapy or tissue-engineered constructs, which are not addressed by standard dressings [70].

Another limitation is the cost associated with advanced wound dressings. While they offer improved outcomes in certain cases, the higher cost of materials such as silver or collagen-based dressings can limit their widespread use, especially in low-resource healthcare settings [71]. In some cases, patients or healthcare systems may opt for less expensive but less effective dressings, which could prolong healing times and increase the overall cost of care due to extended treatment durations.

Additionally, some wound dressings, particularly those designed for long-term use, may not always provide the right balance between moisture management and exudate absorption. Excessively moist environments can lead to maceration of the surrounding skin, while overly

absorptive dressings can dry out the wound, impeding the healing process. Achieving the correct balance requires careful monitoring and frequent dressing changes, which can be time-consuming and labor-intensive for both healthcare providers and patients [72].

1.9. Innovations in wound dressings: focus on nanofibers

Recent advancements in wound dressing technology have increasingly focused on the use of nanofibers, which represent a significant innovation in the field of wound care. Nanofibers, due to their unique properties, offer several advantages over traditional wound dressings, enhancing the overall effectiveness of wound management and promoting faster healing [73].

Nanofibers are characterized by their extremely small diameter, often in the range of nanometers, which endows them with a high surface area-to-volume ratio. This property allows nanofiber-based dressings to provide superior structural support and mimic the natural ECM more effectively than larger fibers or traditional dressings. The high surface area also enhances their capacity for loading and delivering bioactive agents, such as growth factors, antimicrobial agents, or anti-inflammatory drugs, directly to the wound site. This targeted delivery can significantly improve healing outcomes by addressing specific needs of the wound, such as infection control or promotion of cellular proliferation [74-75].

One of the primary innovations in nanofiber wound dressings is the use of electrospinning technology to create these materials. Electrospinning enables the production of nanofibers with controlled diameters and porosity, allowing for the creation of membranes that closely mimic the structure of the ECM. These nanofiber membranes provide an ideal environment for cell attachment and growth, which is crucial for effective tissue regeneration. The fine, porous structure of nanofiber mats also facilitates the efficient exchange of gases and moisture, helping to maintain an optimal wound environment and reduce the risk of maceration or desiccation [73,76].

Incorporating bioactive substances into nanofiber dressings is another significant advancement. For example, nanofibers can be coated or embedded with antimicrobial agents, such as AgNPs or essential oils, to prevent infection and reduce microbial load. This is particularly beneficial in treating chronic wounds, where infection is a common complication. The controlled release of these antimicrobial agents from the nanofiber matrix can provide sustained protection against pathogens, which is essential for managing wounds that are prone to persistent infection [77].

Additionally, nanofiber-based dressings can be engineered to deliver growth factors and other therapeutic agents that enhance the wound healing process. By incorporating substances like collagen, hyaluronic acid, or platelet-derived growth factors into the nanofiber matrix, these dressings can support cellular activities such as proliferation, migration, and ECM production. This approach not only accelerates the healing process but also improves the quality of the regenerated tissue, potentially leading to better functional and cosmetic outcomes [77,78].

Despite their many advantages, nanofiber-based dressings also face some challenges. The complexity and cost of producing these advanced materials can be high, which may limit their accessibility and widespread adoption. Additionally, the long-term safety and efficacy of these dressings in diverse clinical settings need to be thoroughly evaluated through extensive clinical trials.

1.10. Gaps in the literature and opportunities for innovation

Despite significant advancements in wound care and the development of various innovative dressings, several gaps in the literature remain, presenting opportunities for further research and innovation. Identifying and addressing these gaps is crucial for advancing wound management practices and improving patient outcomes.

One notable gap in the literature is the limited understanding of how different types of nanofiber-based dressings interact with various wound environments [79,80]. While much research has focused on the general properties of nanofiber materials and their potential benefits, there is still a need for in-depth studies on how these materials perform in diverse wound types and conditions. For instance, chronic wounds, which often have complex underlying issues such as poor vascularization or persistent infection, may present unique challenges that are not fully addressed by current nanofiber dressings [76,81]. Research into how nanofiber dressings can be optimized for specific wound environments, such as by adjusting their composition or incorporating additional bioactive agents, could lead to more effective solutions tailored to individual patient needs.

Another area where the literature is lacking is in the long-term safety and efficacy of advanced wound dressings, including those based on nanofibers. Most studies focus on short-term outcomes, and there is a need for comprehensive research that evaluates the long-term effects of these dressings on wound healing and tissue regeneration [81,82]. This includes understanding the potential for adverse reactions, such as inflammatory responses or delayed wound healing, and ensuring that the materials used do not pose any long-term risks to patients. Additionally, long-term studies are needed to assess the durability and performance of these dressings in real-world clinical settings, which often involve a range of patient-specific variables.

Cost-effectiveness is another critical gap in the current literature. While innovative dressings, particularly those incorporating advanced materials like nanofibers, show promise, their higher costs can limit their accessibility and widespread use [83,84]. Research into the cost-effectiveness of these dressings compared to traditional options is essential for evaluating their overall value. This includes analyzing not only the initial costs but also the potential for reduced healthcare expenditures due to faster healing times and fewer complications. Developing cost-

effective manufacturing processes and exploring ways to reduce production costs while maintaining high-quality standards could help make these advanced dressings more accessible.

Despite advances in wound healing technologies, there is a notable lack of scientific evidence regarding the influence of nanoroughness in wound dressings on the healing process [79,85]. While it is well-established that surface topography can affect cell behavior, particularly adhesion, proliferation, and differentiation, few studies have specifically explored how nanoscale roughness impacts these processes in wound environments [86-88]. Nanorough surfaces have the potential to mimic the natural ECM, enhancing cellular interactions, yet the precise effects on fibroblast activity, collagen deposition, and overall tissue regeneration remain under-researched [89,90]. The role of nanoroughness in modulating microbial adhesion and biofilm formation is poorly understood, which is critical given the threat of infection in chronic wounds [91].

Furthermore, there is a need for more research on customizing and personalizing wound dressings. While current innovations permit some degree of customization, there is potential to develop dressings tailored to individual patient needs and specific wound characteristics. Advancements in personalized medicine and patient-specific treatment approaches could lead to more effective and individualized wound care solutions. Further investigation is required to establish the relationship between the nanostructure of wound dressings and their ability to promote efficient, infection-free healing.

2. MATERIALS AND METHODS

2.1. Materials used

The selection of materials is a crucial factor in the design and development of advanced wound healing technologies. The ability of these materials to interact harmoniously with biological tissues, support cellular functions, and address common complications such as infection, plays a pivotal role in the overall success of the treatment [64,92]. In this study, the materials were carefully chosen for their biocompatibility, structural properties, and functionality, with a particular focus on enhancing tissue repair and preventing bacterial infections. All products and reagents were of analytical grade, were purchased from Merck (St. Louis, MO USA), and were employed without further purification.

Collagen, a naturally occurring protein in the ECM, was selected as the primary material due to its exceptional ability to promote cell adhesion, migration, and proliferation, key processes in wound healing [40]. Its biocompatibility and structural role in tissue engineering made it a central component of the scaffold. To improve the mechanical properties and durability of the scaffold, synthetic polymers were incorporated, providing the necessary strength while maintaining flexibility and biodegradability.

A significant innovation in this study is the use of AgNPs, known for their potent antimicrobial properties. AgNPs were integrated into the nanofibrous membranes to address the high risk of infection associated with chronic and complex wounds [52]. By leveraging the antimicrobial action of silver, the membrane provides a dual benefit: supporting tissue regeneration while simultaneously preventing bacterial growth, a critical challenge in wound care management.

2.1.1. Collagen

Collagen is a widely utilized biomaterial in tissue engineering, primarily due to its abundance in the ECM and its essential role in supporting cellular processes such as adhesion, proliferation, and tissue repair. Its distinctive triple-helical structure offers mechanical strength and stability, making it an ideal candidate for constructing scaffolds. In addition, collagen has the ability to bind and release growth factors, which enhances its potential to promote localized tissue repair by facilitating cell growth and proliferation [93].

The collagen used in this research was Type I collagen derived from bovine skin. A stock solution was prepared at a concentration of 1 mg/mL (0.1% w/v) by initially dissolving the lyophilized collagen in hydrochloric acid (HCl). The solution was then neutralized with sodium hydroxide (NaOH) and adjusted to physiological pH using phosphate-buffered saline (PBS) to obtain a stable and biocompatible formulation. This concentration was selected based on preliminary tests showing an optimal balance between viscosity, coating uniformity, and bioactivity. The resulting collagen solution exhibited suitable viscosity and stability for surface coating applications following electrospinning. It was subsequently applied onto the plasma-treated PCL nanofiber membranes by drop-casting to enhance bioactivity and promote fibroblast adhesion, mimicking the biochemical and structural characteristics of the native ECM.

2.1.2. Silver Nanoparticles (AgNPs)

AgNPs were a key component of this study, primarily used for their potent antimicrobial properties to combat bacterial infections, a common complication in chronic and complex wounds. To produce the AgNPs, we employed the sodium citrate reduction method, also known as the **Lee-Meisel Synthesis**, a well-established approach for generating AgNPs with controlled size and stability [94].

The Lee-Meisel method is a chemical reduction technique where silver nitrate ($AgNO_3$) is reduced by sodium citrate in an aqueous solution. Sodium citrate serves both as a reducing agent and a stabilizer, preventing the AgNPs from aggregating. During the reaction, silver ions (Ag^+) from silver nitrate are reduced to elemental silver (Ag^0), which then nucleates to form nanoparticles. The citrate ions also stabilize the newly formed nanoparticles by binding to their surface, ensuring they remain well-dispersed in the solution. This method is widely used because it produces AgNPs with a controlled size distribution, which is critical for ensuring consistent antimicrobial activity.

In this study, the concentration of silver nitrate and sodium citrate, along with the reaction conditions (temperature, pH, and stirring rate), were optimized to produce AgNPs of approximately 50-100 nm in diameter. The size of the nanoparticles was chosen based on its relevance to antimicrobial efficacy, as smaller nanoparticles provide a larger surface area for interaction with bacterial cells, enhancing their antibacterial activity [95].

The synthesized AgNPs were incorporated into the electrospun collagen-PCL nanofibrous membranes. Their inclusion aimed to address the issue of wound infection, which can severely impede the healing process. The antimicrobial activity of the AgNPs is attributed to multiple mechanisms: they disrupt bacterial cell membranes, generate ROS that cause oxidative stress, and interfere with bacterial DNA and protein functions, ultimately leading to cell death [26,51]. These multiple attack points make AgNPs particularly effective against a wide range of bacteria, including both gram-positive and gram-negative strains, as well as antibiotic-resistant pathogens like Pseudomonas Aeruginosa and Vancomycin Resistant Enterococcus [96].

One of the advantages of using the Lee-Meisel synthesis is that it allows for the precise control of nanoparticle size and distribution, which is critical for balancing antimicrobial efficacy with cytocompatibility. While AgNPs are effective at killing bacteria, high concentrations can be

cytotoxic to human cells. By carefully controlling the size and concentration of the nanoparticles, we ensured that the silver release was gradual and sustained, providing continuous antimicrobial protection while minimizing any potential cytotoxic effects on the surrounding tissues.

The coating of AgNPs onto the collagen-PCL nanofiber scaffold resulted in a multifunctional wound dressing that not only supports tissue regeneration but also provides long-lasting antimicrobial activity. This dual functionality is particularly valuable in the treatment of chronic and complex wounds, where both infection control and tissue repair are critical to achieving positive healing outcomes.

2.1.3. Polycaprolactone (PCL)

Polycaprolactone (PCL) was selected for this study due to its excellent mechanical properties, biocompatibility, and slow biodegradation rate, which make it an ideal synthetic polymer for tissue engineering and wound healing applications. As a semi-crystalline polymer approved by the U.S. Food and Drug Administration (FDA), PCL provides sufficient mechanical strength and structural support while allowing gradual degradation over time, thereby maintaining scaffold integrity during tissue regeneration [63].

In this project, nanofibrous membranes based on PCL were fabricated using the electrospinning technique, which enables the formation of highly porous fibrous structures that closely resemble the native extracellular matrix (ECM). The high surface area-to-volume ratio and interconnected porosity of electrospun PCL fibers facilitate cell adhesion, proliferation, and nutrient exchange; key processes in tissue repair and wound healing [97].

To prepare the PCL nanofibers, a 12% (w/v) PCL solution was obtained by dissolving 1.2 g of PCL (80,000 Da) in 10 mL of a solvent mixture composed of dichloromethane (DCM) and

dimethylformamide (DMF) in a 7:3 volume ratio. The solution was stirred continuously for 24 h at room temperature to ensure complete polymer dissolution and to achieve the appropriate viscosity and stability for electrospinning.

2.2. Methods for preparing nanofiber membranes

2.2.1. Electrospinning technique and air plasma treatment

Electrospinning was the primary technique used in this study to fabricate nanofiber membranes composed of PCL, collagen, and AgNPs. The process involves applying a high voltage to a polymer solution to create fine nanofibers that are collected on a grounded target plate. This method is highly suitable for creating scaffolds with a structure that closely mimics the ECM, essential for tissue engineering and wound healing [97].

To prepare the membranes, a 12% PCL solution was electrospun under the following parameters:

- **Voltage**: 17 kV
- **Distance between needle tip and receiver plate**: 28 cm
- **Flow rate**: 0.6 ml/h
- **Syringe inner diameter**: 9.1 mm
- **Needle gauge**: 27G
- **Spinning time**: 2 hours

The nanofibers were collected on an aluminum foil-covered static plate and subsequently dried at room temperature for 24 h to ensure complete solvent evaporation.

The random deposition of the electrospun fibers produced a highly porous membrane structure with a large surface area, which is crucial for cell adhesion, proliferation, and migration. After

electrospinning, the membranes were treated with **air plasma** at low pressure (0.13-0.14 atm) to enhance their hydrophilicity [98]. This treatment significantly improved the membrane's ability to absorb the collagen solution during the subsequent coating process and facilitated better cell interaction by increasing surface wettability.

2.2.2. Optimization of coating process with collagen and silver nanoparticles

In addition to PCL, collagen and AgNPs were incorporated into the nanofibrous membranes to enhance biocompatibility and convey antimicrobial properties, respectively. The coating process was carefully optimized to ensure even distribution of both collagen and AgNPs on the surface of the PCL fibers without altering the fiber structure.

Collagen was applied to the PCL nanofibers using a solution of Type I collagen (from bovine skin) diluted in 0.01 M PBS (pH 2) and 0.01 M NaOH. The collagen solution was deposited onto the PCL nanofibers through a drop-coating technique (4ul per 5mm diameter disks), ensuring a thin, even coating that improved the scaffold's hydrophilicity and enhanced cell adhesion. The coated membranes were then allowed to rest for 24 hours before being washed with deionized water to remove any unbound collagen.

AgNPs were synthesized using the Lee-Meisel method, and the resulting particles were incorporated into the membrane to provide antimicrobial properties. The AgNPs were added to the collagen solution and applied via the same drop-coating technique. The concentration of AgNPs (180ng/ul) was optimized to balance antimicrobial efficacy with cytocompatibility, ensuring that the membranes provided effective bacterial inhibition without compromising the scaffold's biocompatibility.

2.3. Membrane characterization

2.3.1. Morphological analysis (SEM, AFM)

To characterize the morphology of the nanofiber membranes, two microscopy techniques were employed: Scanning Electron Microscopy (SEM) and Atomic Force Microscopy (AFM). These methods provided detailed insights into the structure of the membranes, including fiber diameter, surface roughness, and overall morphology.

SEM was used to analyze the surface topography of the electrospun membranes. Samples were mounted on aluminum stub coated with double-side carbon tape. Samples were then coated with carbon using the sputter coater Quorum Q150T ES plus. They were analyzed using a Zeiss Gemini300 SEM equipped with a Bruker XFlash 610M probe for the Energy Dispersive Spectroscopy. The analysis was performed at a working distance of around 8.5 mm. The images were collected using secondary electrons and backscattered electrons using 5 kV of acceleration voltage. The EDS spectra were collected at a magnification of 500X with an acquisition time of 2 minutes, using an acceleration voltage of 10 kV.

SEM works by directing a focused electron beam onto the sample surface, causing the emission of secondary electrons. These electrons are collected to generate high-resolution images of the membrane's surface. SEM was critical for assessing the nanofiber diameter, distribution, and porosity of the scaffold, ensuring that the electrospinning process yielded consistent and uniform fibers. Additionally, SEM allowed for the evaluation of whether the collagen and AgNPs coatings had any significant effect on the surface morphology of the fibers.

AFM was utilized to provide more detailed information about the surface roughness and nanoscale topography of the membranes. Samples were mounted on a magnetic sample holder using double-sided adhesive tape. The analysis was performed using a Bruker Dimension Icon

AFM equipped with an antimony-doped silicon tip (SCM-PIT-V2, Bruker) in semi-contact mode. Scans were conducted over areas of 10 × 10 μm and 5 × 5 μm with a scan rate between 0.8 and 0.3 Hz. Surface topography was captured under ambient conditions at a resolution of 512 × 512 pixels. Post-processing, including leveling and surface roughness analysis, was completed using Bruker NanoScope Analysis software. Measurements were repeated in triplicate across different regions of the sample to ensure data reliability.

AFM operates by scanning a sharp tip across the surface of the sample, measuring the forces between the tip and the surface to generate a three-dimensional profile. This technique was particularly important for evaluating the effect of collagen coating and AgNPs on the nanofiber surface. AFM allowed for the quantification of changes in surface roughness, which is crucial for understanding how these modifications may influence cell adhesion and migration on the membrane. Additionally, AFM helped to visualize the nanoscale distribution of the AgNPs across the surface of the fibers, ensuring their uniform incorporation.

The combined use of SEM and AFM provided a comprehensive characterization of the membrane's morphology, ensuring that the electrospinning and coating processes produced nanofibers with the desired structural properties for biomedical applications.

2.3.2. Mechanical and physical properties

The mechanical and physical properties of the electrospun nanofiber membranes were critical to ensuring their effectiveness in wound healing applications, particularly with regard to structural integrity, flexibility, and degradation behavior. To assess these characteristics, several key evaluations were conducted, including tests for tensile strength, water absorption, and degradation.

Tensile strength testing was performed to measure the mechanical resilience and flexibility of the nanofiber membranes. This involved applying uniaxial tensile stress to the membranes using a universal testing machine, which continued until the samples ruptured. The force and elongation at failure were recorded, allowing for the calculation of parameters such as ultimate tensile strength (UTS), Young's modulus, and elongation at break. These mechanical properties are vital for ensuring that the membranes can endure the mechanical stresses present at the wound site, while also being flexible enough to adapt to the shape and movement of the skin and body parts. PCL provided the structural integrity required for durability, while the collagen coating enhanced flexibility and biocompatibility.

The membranes' hydrophilicity and water absorption capacity were also evaluated, as these properties are essential for managing fluid accumulation at the wound site. The ability to absorb water was enhanced through air plasma treatment, which increased the surface wettability of the membrane. By immersing the membranes in saline water and monitoring weight changes, it was possible to determine the membrane's capacity to absorb moisture according to the equation:

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

This hydrophilic behavior is important for maintaining a moist wound environment, which is conducive to faster tissue regeneration and overall healing.

2.4. In vitro biological evaluation

2.4.1. Biocompatibility testing

Biocompatibility is a crucial factor in the design of biomaterials intended for use in tissue engineering, particularly in wound healing applications. The primary objective of biocompatibility testing is to ensure that the material can support cellular processes such as adhesion, proliferation, and migration without inducing any adverse biological responses, such as inflammation or immune rejection. In this study, a comprehensive biocompatibility evaluation was performed to confirm that the electrospun nanofiber membranes, functionalized with collagen and AgNPs, could provide a conducive environment for cell growth and tissue regeneration.

Cell model and preparation

3T3 murine fibroblasts were selected as a pragmatic model of dermal repair given their reproducible growth and central role in extracellular matrix deposition. Prior to seeding, 5-mm discs (PCL, PCL+collagen, PCL+collagen+citrate as negative control and PCL+collagen+AgNPs)—were UV-sterilized and pre-conditioned in complete medium to stabilize the solid–liquid interface and minimize artefacts from initial protein adsorption.

Culture conditions

Cells were maintained in high-glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C, 5% CO_2, ≥95% humidity. Discs were placed in multi-well plates and equilibrated for 30 min before cell addition.

Adhesion and morphology

The ability of the fibroblasts to adhere to the nanofiber membranes is an important indicator of biocompatibility, as proper adhesion is necessary for subsequent cellular functions like proliferation and migration [99]. Early attachment and spreading were examined by a dual imaging strategy. SEM and Fluorescence Microscopy (DAPI for nuclear stain and phalloidin for F-actin) imaging was used to observe cell attachment to the membrane surface

MTT proliferation and viability assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) assay was performed at each time point (24, 48, and 72 hours). The MTT assay is a colorimetric test that measures the metabolic activity of viable cells. It works by introducing a tetrazolium salt, which is metabolized by mitochondrial enzymes in living cells to produce insoluble formazan crystals. The amount of formazan produced is directly proportional to the number of viable cells present on the membrane. After incubation with the MTT reagent, the formazan crystals were solubilized in DMSO, and the absorbance was measured at 570 nm using a microplate reader. Higher absorbance values indicated greater cell viability and proliferation [100-101].

Alamar Blue assay for proliferation (on-disc model)

Proliferation/viability was quantified at 24h, 72h and 7days using Alamar Blue with cells seeded directly on the membrane discs; between time points only the culture medium was replaced to enable non-destructive longitudinal measurements. At each time point, medium was exchanged for resazurin 1:30 (v/v) in phenol-red–free DMEM and samples were incubated 3–4 h at 37 °C protected from light. Supernatants were transferred to a black 96-well plate for fluorescence reading (Ex 560 nm / Em 590 nm) or to a clear plate for absorbance at 570 nm with 600 nm reference. Each plate included: (i) medium blanks, (ii) disc+reagent blanks without

cells to correct any optical contribution from the materials/AgNPs, and (iii) cell-only controls on tissue-culture plastic as the 100% viability reference. Signals were blank-subtracted and normalized to cell-only controls; the percentage reduction of resazurin was calculated according to the supplier's equations. Linearity was verified with low-density cell controls to confirm the instrument's dynamic range. Because the assay is non-lytic, discs were returned to fresh medium after reading to continue incubation to the next time point. Results were expressed as relative fluorescence units (RFU) normalized to cell-only controls, and for absorbance the dual-wavelength equation was applied. Higher RFU (or blank-corrected A570/A600) indicated greater numbers of viable, proliferating cells; potential nanoparticle interference was controlled

2.4.2. Antibacterial activity assessment

Preventing bacterial and fungal infections is essential in wound healing, as infections can greatly limit the healing process and lead to severe complications. In this study, the antibacterial and antifungal properties of the nanofiber membranes functionalized with AgNPs were evaluated to determine their effectiveness against a range of pathogens. AgNPs were chosen for their well-known broad-spectrum antimicrobial activity, which is effective against both bacteria and fungi, including drug-resistant strains [102].

Broth microdilution test

AgNPs Antimicrobial activity was assessed against three biofilm-forming strains relevant to wound infection: Staphylococcus aureus ATCC 29213 (SA) [103-104], a vancomycin-resistant Enterococcus faecium clinical isolate (VRE, Ef-5) [105] and Pseudomonas aeruginosa ATCC 27853 (PA) [106]. To avoid confounding from silver released by the membranes, intrinsic susceptibility to citrate-stabilized AgNPs was established in liquid culture prior to on-surface testing. Minimum inhibitory concentrations (MICs) were determined by standard microbroth dilution in cation-adjusted Mueller–Hinton broth (CAMHB, Oxoid) in accordance with Clinical

Laboratory Standards Institute (CLSI) guidelines [107]. AgNPs suspensions were prepared up to 400 μg/mL and subjected to two-fold serial dilution in 96-well plates. Log-phase bacteria were adjusted to an initial inoculum of 5×10^5 CFU/mL and incubated with AgNPs at 37 °C for 20 h. Growth was evaluated by visual assessment of turbidity against appropriate controls, and the MIC was defined as the lowest AgNP concentration that completely suppressed visible growth. Resulting MIC values served as quantitative benchmarks for interpreting subsequent membrane-based assays.

Biofilm formation inhibition

Biofilm inhibition is crucial for reducing chronic wound infections, which are notoriously difficult to treat due to the protective nature of biofilms. [108]. To capture early surface colonization, 5-mm discs (PCL, PCL+collagen, PCL+collagen+AgNPs) were incubated for 24 h at 37 °C, 200 rpm with 10^7 CFU/mL in Luria-Bertani broth (*P. aeruginosa*) or Brain Heart Infusion (*S. aureus*, VRE). After gentle PBS rinses to remove planktonic cells, adherent biomass was fixed in methanol, stained with 2% crystal violet, and eluted with 33% acetic acid for spectrophotometry at 560–570 nm. Results were reported both as raw absorbance and as percentage reduction relative to PCL, isolating the incremental effect of collagen and the specific contribution of AgNPs.

2.4.3. Cytotoxicity testing

Cytotoxicity was assessed with two complementary readouts: Alamar Blue under a contact-exposure (suspension) model and LDH release from supernatants. Unlike the proliferation assay, where cells were seeded directly on the discs and only the culture medium was changed, the cytotoxicity protocol exposed suspended cells to the membranes and quantified death after contact.

Alamar Blue cytotoxicity (contact-exposure, suspension model)

Cells in suspension were added to wells containing the test discs and incubated for the predefined contact period (24 h; 37 °C, 5% CO_2). At endpoints (24h and 48h), discs were removed to avoid optical interference. Cell suspensions were gently mixed and transferred to assay plates.

Alamar Blue working solution (resazurin, 1:30 v/v in phenol-red–free DMEM) was added and incubated 3–4 h at 37 °C, protected from light. Fluorescence was read at Ex 560 nm/Em 590 nm.

Each plate contained:

- medium blanks;
- disc+reagent blanks (disc incubated without cells, then removed before reading) to correct residual contributions from released species;
- cell-only controls (no disc) as 100% viability reference.

Percent viability was calculated using blank-subtracted signals and normalized to cell-only controls. Percent cytotoxicity was reported as 100-viability. Linearity was verified with low-density controls. Because readings were taken after disc removal, interference from AgNPs or dye adsorption on the substrate was minimized and further corrected by the disc+reagent blanks.

LDH release test

In parallel wells, supernatants collected after the same contact period (24h and 48h) were processed with an LDH kit. Absorbance was measured at 490 nm with a 600–620 nm reference.

Three controls were included:

- spontaneous LDH (low control);
- +detergent-lysed cells (high control);
- interference controls (reagent only; disc+reagent without cells).

Cytotoxicity (%) was computed as:

$$\frac{\text{Sample} - \text{Low}}{\text{High} - \text{Low}} \times 100.$$

Values ≤10–15% were interpreted as low acute cytotoxicity under the assay conditions.

2.4.4. Statistical analysis

Data analysis was planned a priori to compare membrane formulations on antibacterial and cytocompatibility outcomes. For biofilm inhibition, four independent biological replicates per condition were acquired; technical replicates, when present, were averaged within replicate. For each bacterial strain, a one-way ANOVA tested the effect of membrane type, followed by Tukey's HSD for pairwise contrasts. For cell viability, MTT data were analysed separately at 24, 48, and 72 h using one-way ANOVA with Tukey's HSD when the omnibus test was significant. For the cytotoxicity workflow, Alamar Blue (suspension model) and LDH release were analysed at 24 and 48 h by one-way ANOVA with Tukey's HSD at each endpoint. Normality (Shapiro–Wilk) and homogeneity of variances (Levene's test) were verified; when assumptions were violated, data were inspected for outliers and log or square-root transformation was considered before re-analysis. Results are reported as mean ± SD; two-sided tests were used with $\alpha = 0.05$. Where relevant, η^2 (effect size) and adjusted p-values from Tukey's procedure are provided. MIC determinations were treated as descriptive endpoints. All analyses were performed in Python 3.11 using SciPy and statsmodels.

3. RESULTS

3.1. Membrane characterization

The characterization of the nanofiber membranes was essential to confirm that the electrospinning process, along with the incorporation of collagen and AgNPs, resulted in a structure suitable for wound healing applications. The results provided insights into the morphology, mechanical properties, and surface characteristics of the membranes.

3.1.1. Morphological analysis

SEM revealed that the electrospun nanofiber membranes had a uniform, porous structure with fibers randomly arranged, mimicking the ECM. The average fiber diameters ranged from approximately 350 to 550 nm (Figure 1), consistent with the optimized electrospinning parameters. The SEM images also showed that the collagen coating was evenly distributed over the PCL fibers without significantly altering their morphology. The collagen layer enhanced the bioactivity of the membrane while maintaining its structural integrity. Additionally, the incorporation of AgNPs did not cause any noticeable aggregation or disruption to the fiber structure.

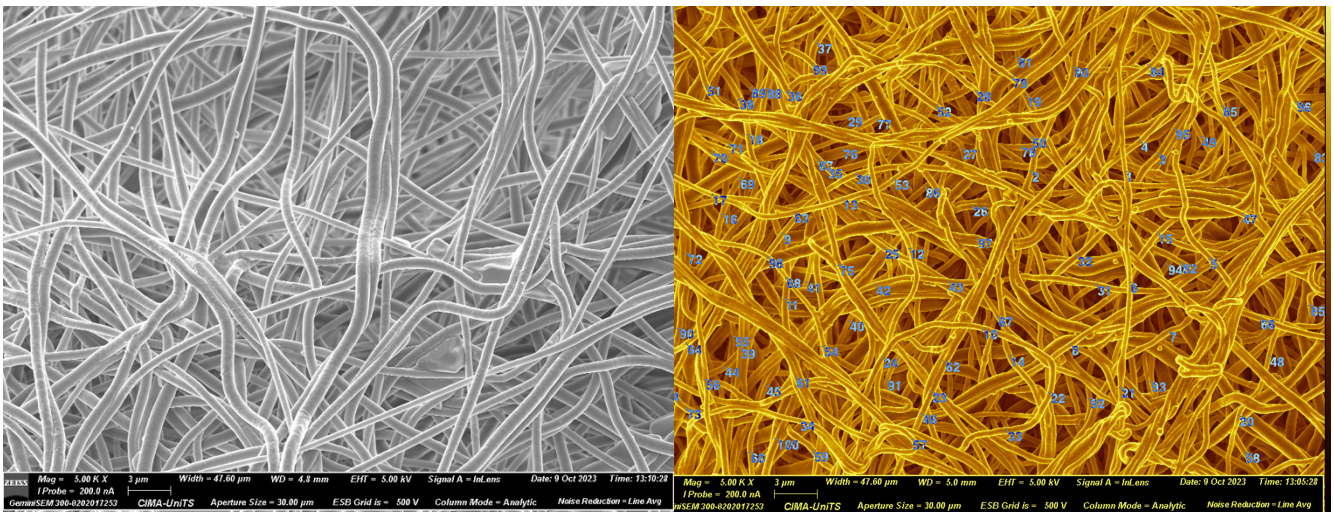
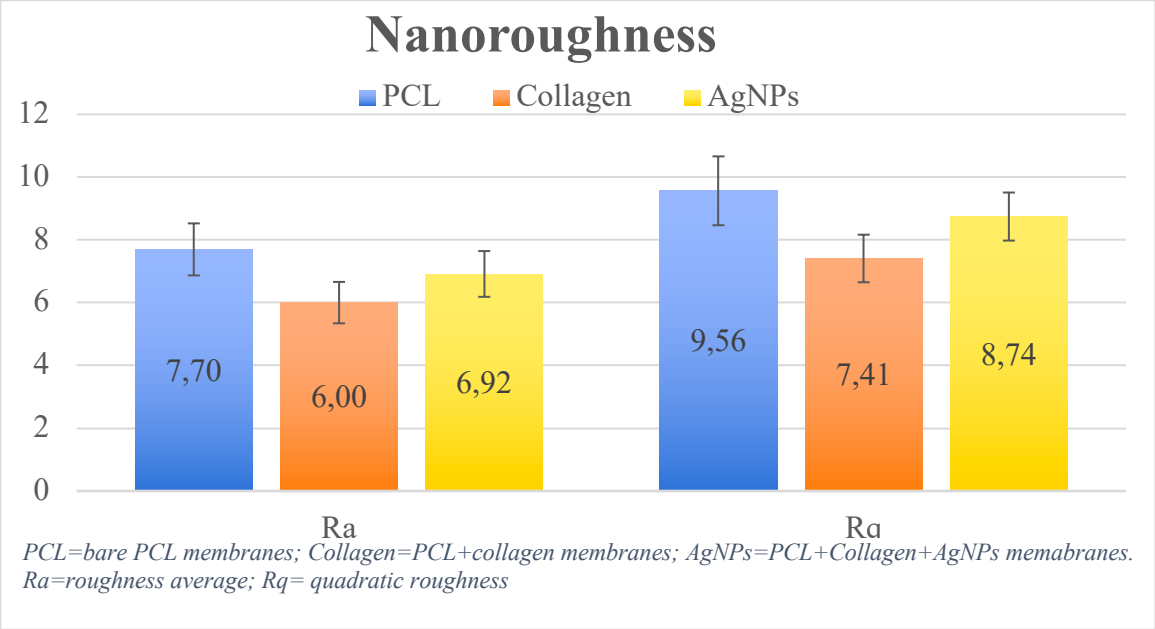


Figure 1: Nanofibers morphology and arrangement at SEM imaging

AFM further provided details about the surface roughness of the membranes. AFM analysis showed that the collagen coating decreased the surface roughness of the fibers, AgNPs increased it. Data of nanoroughness are shown in Figure 2. AFM allowed us also to measure the size of AgNPS that was confirmed to be 50-100nm.



PCL=bare PCL membranes; Collagen=PCL+collagen membranes; AgNPs=PCL+Collagen+AgNPs memabranes. Ra=roughness average; Rq= quadratic roughness

Figure 2: AFM fibers nanoroughness

3.1.2. Mechanical properties

Mechanical testing used dog-bone–shaped specimens. Nevertheless, the small gauge dimensions approached the lower handling limits of the universal testing machine, leading to alignment and gripping constraints that introduced variability and hampered full standardization. Despite these limitations, the Young's modulus (consistently indicated a compliant material rather than a rigid film. No substantial differences were observed among bare PCL, collagen-coated PCL, and AgNP-functionalized PCL, nor after material aging up to 6 months. Tensile behavior remained stable across groups, with high extensibility at failure (mean strain at break ≈ 237%), compatible with body movements at the wound site.

3.1.3. Hydrophilicity and water absorption

Surface hydrophilicity is an important factor for promoting cell attachment and fluid absorption in wound dressings [23]. The **contact angle measurements** conducted on the membranes demonstrated that the air plasma treatment significantly improved their hydrophilicity (Figure 3). The contact angle started from around 140° and was reduced after plasma treatment (32°), indicating an increase in surface wettability, which was further enhanced by the collagen coating (around 0°).

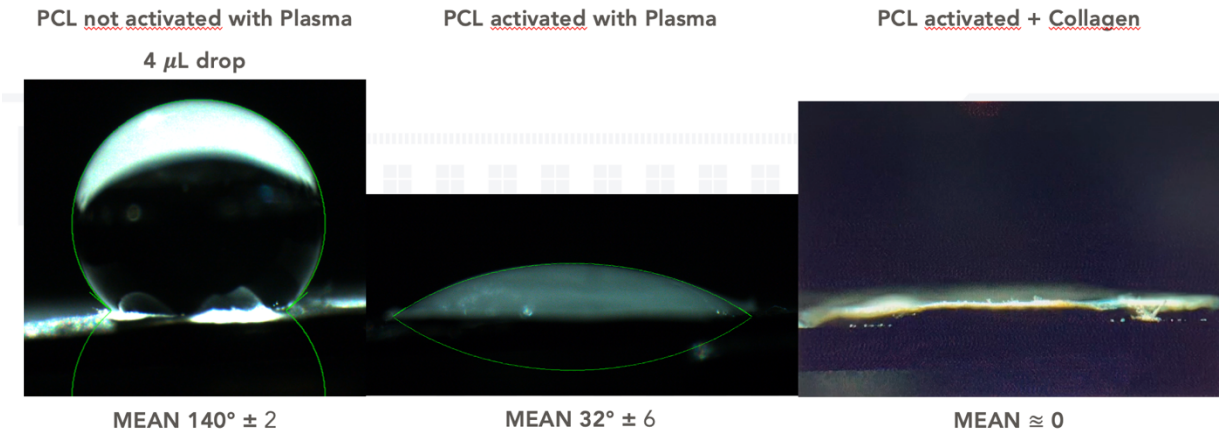


Figure 3: water contact angle of membranes

Water absorption tests confirmed that the air-plasma activated membranes exhibited excellent fluid retention capacity, passing from 53% ± 4 to 140% ± 5.

3.2. Biocompatibility

3.2.1 MTT results

MTT Assay results (Figure 4) demonstrated high cell viability across all membranes, particularly those functionalized with AgNPs. After 24, 48, and 72 hours, cell proliferation increased steadily, with the PCL-collagen and PCL-collagen-AgNPs membranes showing comparable or higher levels of viability compared to the control (PCL-only membranes). Notably, at 72 hours, the AgNPs-functionalized membranes exhibited a significantly higher rate

of cell proliferation, suggesting that the AgNPs did not hinder cell growth. These results indicate that the membranes provide an appropriate environment for cell adhesion and proliferation, which is essential for wound healing.

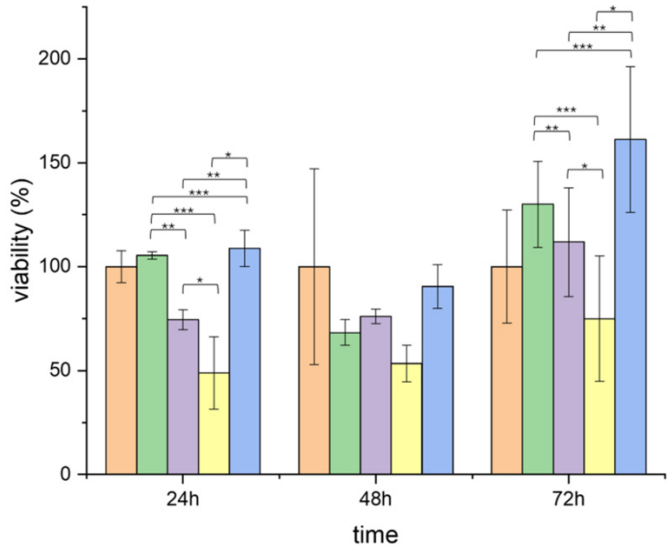


*Figure 4: Cell viability expressed as a percentage relative to the positive control of cell growth (orange) for cells treated with PCL (green), PCL-col (violet), PCL-col-citrate (yellow), and PCL-col-citrate-AgNPs (blue) membranes. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.*

SEM analyses confirmed that fibroblasts adhered well to the surfaces of the nanofiber membranes. The cells exhibited a typical healthy morphology, spreading across the fibers with well-developed filopodia.

3.2.1 Alamar blue proliferation results

Alamar Blue proliferation assays showed a monotonic increase in metabolic activity from day 1 to day 7 across all membranes, with low baseline signals converging at day 1 and progressive divergence thereafter (Figure 5). By day 3, AgNP-functionalized discs exceeded collagen-coated and bare PCL, and this ranking persisted at day 7. At the final time point, AgNPs-functionalized discs displayed the highest fluorescence values, PCL+COLL was intermediate, and bare PCL remained the lowest, indicating the weakest proliferative support among the

groups. Fold-change analysis relative to day 1 (Figure 6) confirmed this pattern: AgNPs samples showed the greatest expansion by day 7, followed by PCL+COLL+CIT, PCL+COLL, and bare PCL. Error bars indicated limited intra-group variability, supporting the robustness of the trend. Overall, collagen coating improved proliferation over PCL, and surface functionalization with AgNPs further enhanced the longitudinal increase in viable cell signal.

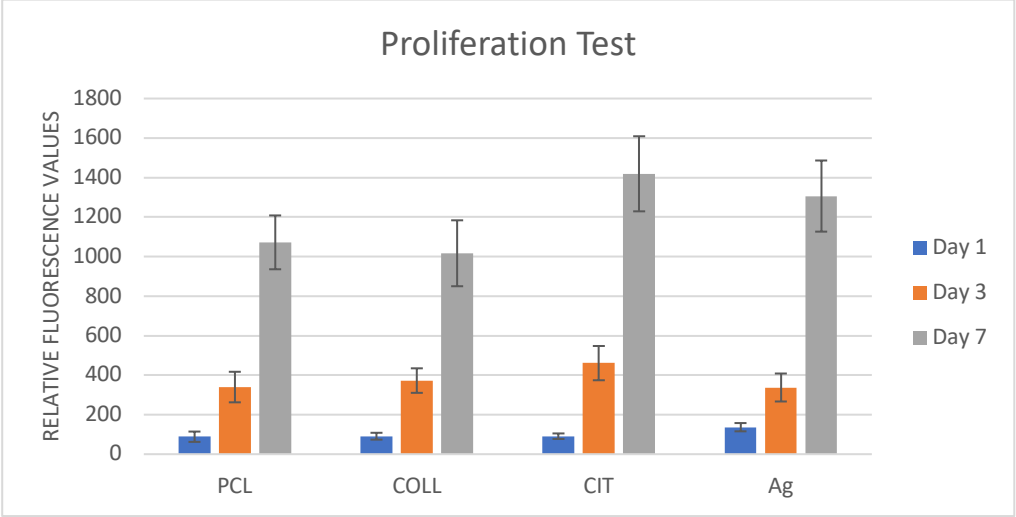


Figure 5: Alamar blue proliferation on membranes at days 1,3 and 7.

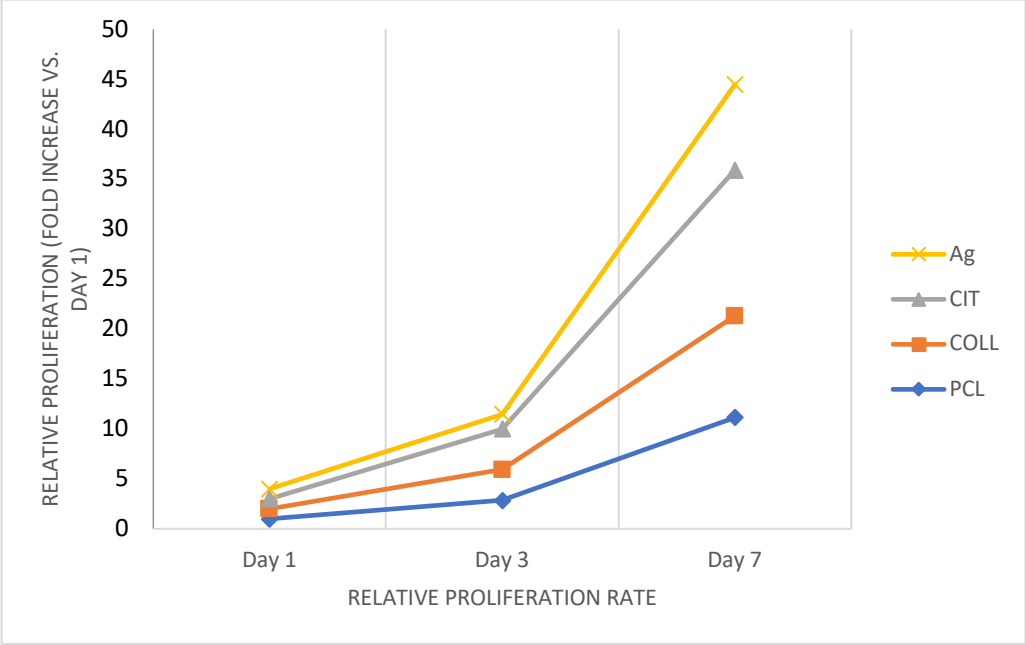


Figure 6: Alamar Blue proliferation expressed as fold increase relative to day 1 at days 1, 3 and 7.

3.3. Antibacterial activity evaluation

The antibacterial properties of the nanofiber membranes functionalized with AgNPs were evaluated through broth dilution and biofilm formation inhibition tests. These tests aimed to determine the effectiveness of the membranes against both gram-positive and gram-negative bacteria strains relevant to wound infections.

3.3.1 Microbroth dilution test

The broth dilution test was performed to determine the MIC of the AgNPs against the tested microbial strains. MIC represents the lowest concentration of an antimicrobial agent that prevents visible bacterial growth.

The AgNPs-functionalized membranes were tested in broth media against the same bacterial strains (SA, VRE and PA). The MIC values obtained were:

- PA: 50 µg/mL
- VRE: 100 µg/mL
- SA: >400 µg/mL.

These results show that Pseudomonas Aeruginosa was particularly susceptible to the AgNPs, with a MIC of 50 µg/mL. VRE demonstrated moderate sensitivity, while Staphylococcus Aureus was resistant even at higher concentrations, exceeding 400 µg/mL. The AgNPs concentration used in the membranes (180 µg/mL) was sufficient to inhibit PA and VRE growth but less effective against SA.

3.3.2 Biofilm formation inhibition

The AgNPs-functionalized membranes exhibited significant biofilm inhibition, particularly against Pseudomonas Aeruginosa and Vancomycin-resistant Enterococcus. Indeed, biofilm reduction when compared to non-functionalized membranes was 39.74% for PA, 55.11% for VRE. In contrast, biofilm inhibition against Staphylococcus Aureus was moderate 30.75%, aligning with the MIC, which showed less susceptibility to the AgNPs. Results of biofilm inhibition are shown in Figure 7.

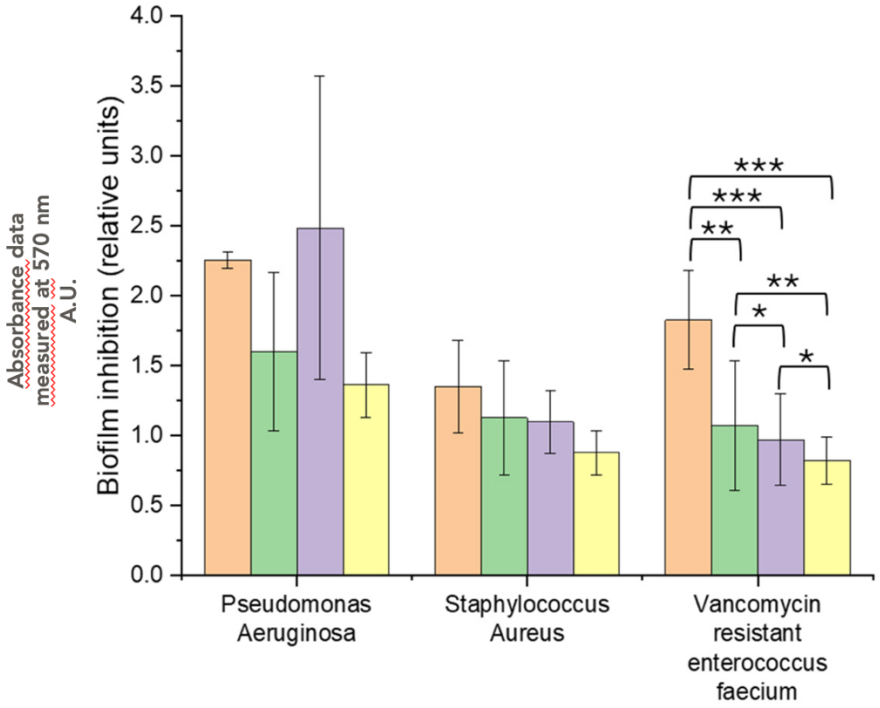


Figure 7: Inhibition of biofilm growth, derived from absorbance measurements in the presence of PCL(orange), PCL-col (green), PCL-col-citrate (violet), and PCL-col-citrate-AgNPs (yellow) membranes.
**: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$*

3.4. Citotoxicity

Cytotoxicity testing focused on evaluating the safety of the AgNPs-functionalized membranes, as excessive AgNPs concentrations can induce cytotoxic effects through the generation of ROS [109].

3.4.1 Alamar Blue cytotoxicity test

In the Alamar Blue cytotoxicity suspension model (Figure 8), viability normalized to the cell-only control exceeded 100% at 24 h for all conditions, with the ranking Ag > PCL >> Cit ≳ Col. At 48 h, signals decreased across groups but the order remained essentially unchanged (Ag highest; PCL ≈ Cit ≥ Col). The pattern indicates no acute cytotoxicity after 24-48h of contact and overall good tolerability of all coatings, with silver-functionalized membranes sustaining the highest viable signal at both endpoints.

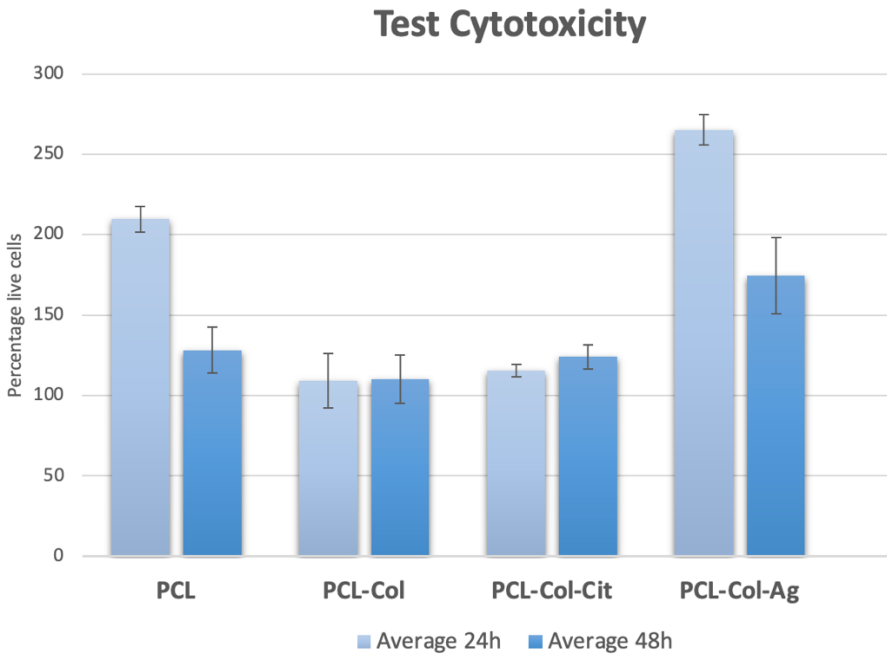


Figure 8: Alamar Blue citotoxicity suspension model test

3.4.1 LDH cytotoxicity test

Cytotoxicity remained low-to-moderate across all membranes and far below the positive control (Figure 9). Mean LDH release at 24 h was ~21% for untreated cells, ~22% for PCL, ~21% for Col, ~24% for Cit, and ~34% for Ag (dead-cell control ≈100%). At 48 h, values decreased or stabilized: ~11% (untreated), ~15% (PCL), ~17% (PCL-Col), ~20% (PCL-Col-Cit), and ~32% (PCL-Col-Ag). Ag showed the highest but still submaximal LDH release. Overall, results indicate no acute cytotoxicity**,** with a slight time-dependent reduction in LDH for most groups and stable, moderate levels for the silver-functionalized membranes.

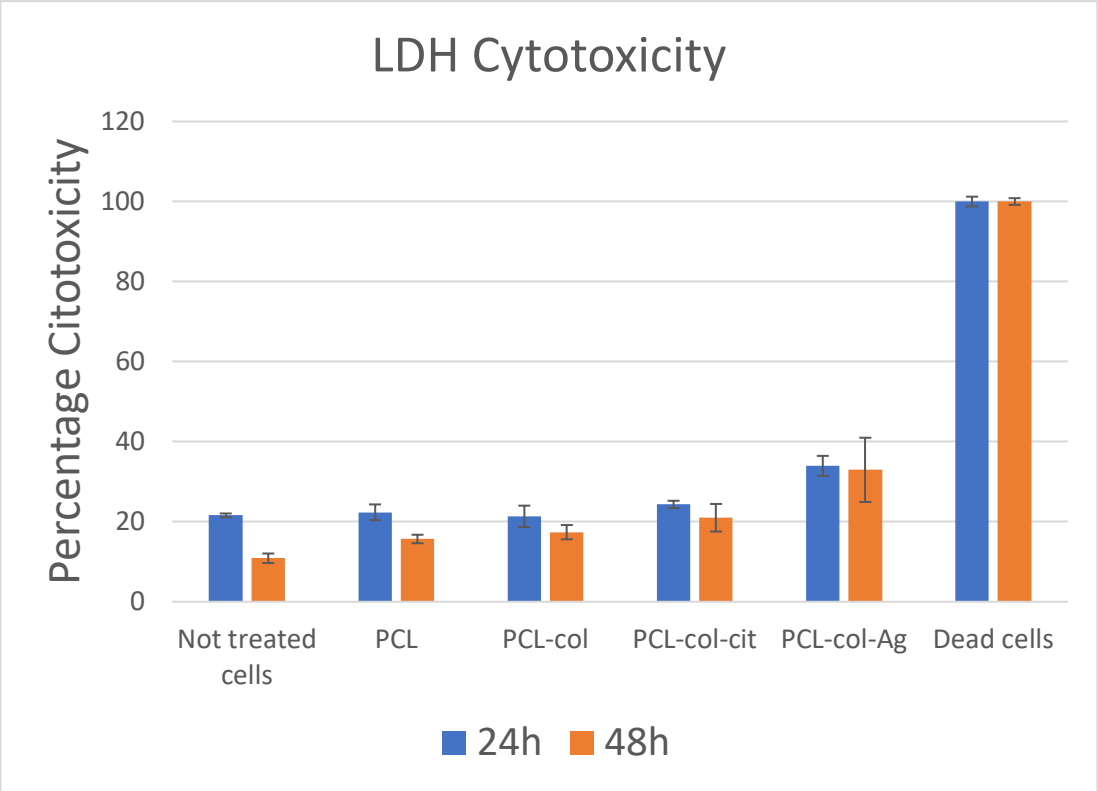


Figure 9: LDH Citotoxicity test

4. DISCUSSION

4.1. Interpretation of results

The characterization, biological evaluation, and antibacterial activity assessment of the nanofiber membranes revealed several key findings that highlight the potential of these materials for advanced wound healing applications. The combination of PCL, collagen, and AgNPs resulted in multifunctional scaffolds that provide both structural support and antimicrobial protection, while maintaining biocompatibility with 3T3 cells.

4.1.1. Morphological and mechanical properties

The SEM analysis demonstrated that the electrospun nanofiber membranes successfully replicated the structure of the ECM, with uniformly distributed, porous fibers arranged in a random fashion. This arrangement is ideal for promoting cell attachment and proliferation, as it mimics the natural architecture of the ECM, providing a conducive environment for tissue regeneration [110]. The measured fiber diameters, ranging between 350 to 550 nm, align with the expected outcomes from the optimized electrospinning parameters, indicating consistent fabrication quality.

Furthermore, the SEM images revealed that the collagen coating was uniformly applied to the PCL fibers, without altering their morphology. This suggests that the collagen enhanced the bioactivity of the membranes, potentially improving cellular responses, while preserving the structural integrity essential for wound healing applications [111]. Importantly, the incorporation of AgNPs into the membrane did not lead to nanoparticle aggregation or disruption of the fiber architecture. This indicates successful integration of the AgNPs into the nanofiber matrix, maintaining the desired structural characteristics while potentially enhancing the membrane's antibacterial properties without compromising its mechanical stability [112].

The AFM analysis provided key insights into the nanostructural modifications of the electrospun membranes. The reduction in surface roughness following collagen coating indicates that the collagen layer smoothened the fiber surface, likely creating a more uniform and continuous covering over the PCL fibers. This smoother surface could enhance cell adhesion and proliferation, as lower surface roughness is known to promote more stable cell-material interactions [113]. In contrast, the presence of AgNPs significantly increased the surface roughness, suggesting that the nanoparticles disrupted the smooth collagen layer and created additional topographical features [114]. This increase in nanoroughness could enhance the antibacterial properties of the membrane, as higher surface roughness has been correlated with increased bacterial inhibition [115]. Moreover, the AFM confirmed that the AgNPs incorporated into the membrane had a size range of 50-100 nm, which is consistent with the effective size range for nanoparticles to exert strong antibacterial effects while maintaining biocompatibility. These findings support the dual role of collagen and AgNPs in optimizing both biocompatibility and antibacterial activity through modulation of surface topography.

The mechanical testing results indicated that the nanofiber membranes exhibited sufficient tensile strength and flexibility to be used as wound dressings. The balance between stiffness and flexibility is critical for wound healing applications, where the scaffold must be able to conform to the wound site while maintaining enough strength to support cell proliferation and tissue integration.

4.1.2. Biocompatibility and cytotoxicity

The biocompatibility assessment using 3T3 murine fibroblasts demonstrated that the nanofiber membranes provided a supportive environment for cell growth, which is crucial for wound healing applications [2].

The **MTT assay** results showed high cell viability across all tested membranes, including those functionalized with collagen and AgNPs. Remarkably, the membranes incorporating AgNPs exhibited the highest levels of cell proliferation at 72 hours, indicating that the presence of AgNPs did not inhibit cell growth. Instead, they may have contributed to an environment conducive to cell proliferation, further enhancing the bioactivity of the membranes. The COLL and Ag membranes demonstrated comparable or even superior cell viability to the control (bare PCL membranes), which underscores the importance of collagen in promoting cellular responses. This suggests that these functionalized membranes could be beneficial for wound healing by facilitating tissue regeneration while also potentially providing antibacterial properties due to the presence of AgNPs.

The **on-disc Alamar Blue assay** showed a progressive rise in metabolic signal from day 1 to day 7 for all membranes, with a stable hierarchy Ag > CIT > COLL > PCL. The gain on COLL versus bare PCL aligns with ECM-like cues and increased hydrophilicity after coating, which favor adhesion and spreading. The additional increase on citrate- and AgNP-functionalized surfaces is plausibly related to altered protein adsorption and surface charge that further support early cell activity; this remains inferential. Overall, the data indicate that collagen enhances initial cell establishment and that AgNPs functionalization, at the applied loading, does not impair and may augment the longitudinal increase in viable signal.

The cytotoxicity evaluation of the AgNPs-functionalized membranes provided crucial insights into their safety profile, particularly given the known risks of AgNPs at high concentrations, which can generate ROS and induce cytotoxicity [25]. The **MTT assay** demonstrated that the AgNPs did not induce significant cytotoxic effects on fibroblast cells, showing minimal cytotoxicity across all time points (24, 48, and 72 hours). Cell viability remained close to 100% after 24 hours of exposure to the AgNPs-functionalized membranes, and this trend persisted through 48 and 72 hours.

In the **suspension Alamar Blue assay**, viability exceeded 100% of cell-only controls at 24 h for every group and, despite a general decline at 48 h, remained high; this argues against acute, contact-mediated toxicity. **LDH release** was low to moderate at both endpoints and far below the dead-cell control, with the highest values observed for the silver-functionalized membrane but without concordant loss of metabolic activity. The agreement between preserved Alamar Blue signal and submaximal LDH supports intact cell function and membrane integrity at the applied AgNPs loading. Overall, the data support cytocompatibility of all formulations over 24-48 h, with a small, noncritical membrane stress attributable to silver. Future work should pair these endpoints with apoptosis/necrosis markers and ROS assays to consolidate mechanism and margins of safety.

These findings are particularly critical as AgNPs, known for their antimicrobial activity via ROS generation, could potentially harm mammalian cells if used at excessively high concentrations [116]. However, the concentration of AgNPs incorporated into these membranes was carefully optimized to provide antibacterial effects without compromising fibroblast viability, as demonstrated by the citotoxicity results. The careful balance between antimicrobial efficacy and cytocompatibility is one of the main strengths of these membranes, making them promising candidates for wound healing applications where both infection control and tissue regeneration are essential.

4.1.3. Antibacterial activity

The **broth dilution test** provided valuable insights into the antimicrobial efficacy of AgNPs-functionalized membranes by determining the MIC against key bacterial strains. The results revealed that *Pseudomonas aeruginosa* (PA) was highly susceptible to AgNPs, with a MIC of 50 µg/mL, indicating that low concentrations were sufficient to inhibit its growth. *Vancomycin-*

resistant Enterococcus (VRE) showed moderate sensitivity, with a MIC of 100 µg/mL, while *Staphylococcus aureus* (SA) exhibited significant resistance, with a MIC exceeding 400 µg/mL.

These findings suggest that the AgNPs concentration used in the membranes (180 µg/mL) was effective at inhibiting the growth of PA and VRE but less effective against SA, even at higher concentrations. The particular susceptibility of PA to AgNPs makes these membranes especially useful for applications where PA infections are prevalent. However, the reduced efficacy against SA indicates that this strain exhibited resistance to the AgNPs, and it may be due to the development of specific mechanisms by SA, therefore additional strategies, such as combining AgNPs with other antimicrobial agents, may be needed to address infections involving SA [117][118]. Overall, the selective antimicrobial activity highlights the potential of AgNPs-functionalized membranes in targeting specific bacterial strains while maintaining a balance between antimicrobial potency and biocompatibility.

Furthermore AgNPs-functionalized membranes demonstrated significant **biofilm inhibition**, particularly against *Pseudomonas aeruginosa* (PA) and *Vancomycin-resistant Enterococcus* (VRE). The reduction in biofilm formation compared to non-functionalized membranes was substantial, with decreases of 39.74% for PA and 55.11% for VRE. These findings highlight the strong efficacy of the AgNPs in disrupting biofilm formation, particularly against PA and VRE, both of which pose significant risks in chronic wound infections. However, the biofilm inhibition against *Staphylococcus aureus* (SA) was more moderate at 30.75%, consistent with the MIC results that showed SA was less susceptible to the AgNPs. This suggests that while the AgNPs-functionalized membranes are effective in preventing biofilm formation for most tested strains, further optimization might be required to enhance their performance against more resistant bacteria like SA. The ability of the membranes to inhibit biofilm formation is critical in addressing chronic wound infections, as biofilms are known to shield pathogens from both immune responses and antimicrobial treatments, complicating wound management [108].

4.2. Comparison with existing literature

The development of nanofiber membranes functionalized with AgNPs for wound healing applications aligns with recent trends in biomaterial research, where multifunctional scaffolds are designed to address the dual challenges of promoting tissue regeneration and preventing infection. The results of this study show that the membranes developed exhibit significant potential in these areas, but it is important to compare these findings with existing literature to contextualize the results and highlight both the advancements and limitations relative to other approaches.

4.2.1. Antibacterial efficacy

The incorporation of AgNPs into nanofibrous scaffolds as an antimicrobial agent has been extensively studied due to the well-documented ability of silver to disrupt bacterial cell membranes, generate ROS, and interfere with microbial DNA replication. In the present study, the AgNPs-functionalized membranes demonstrated strong antibacterial activity against PA and VRE. These findings are consistent with previous research, where AgNPs have been shown to effectively inhibit a wide range of gram-negative bacteria, including PA, a highly antibiotic-resistant pathogen frequently found in chronic wounds [117].

Similar to our findings, other studies have shown that SA can exhibit varying levels of resistance to AgNPs, especially at higher bacterial concentrations or in biofilm-forming conditions. Research suggests that SA may develop protective mechanisms against silver, such as efflux pumps or biofilm-specific phenotypes [118], which can limit the effectiveness of AgNPs. Comparatively, our study demonstrated reduced efficacy of AgNPs against SA, reinforcing the need for further optimization or combination therapies to enhance antibacterial effects against this pathogen. This aligns with other research that suggests combining AgNPs

with antibiotics or other antimicrobial agents can broaden the antimicrobial spectrum and overcome resistance mechanisms [119].

4.2.2. Biofilm inhibition

Biofilm formation is a critical barrier in wound healing, and addressing biofilm-related infections is a key area of focus in wound care research. Several studies have highlighted the role of AgNPs in preventing biofilm formation [120-123], particularly against gram-negative bacteria like PA, which is known for its strong biofilm-forming capabilities. The current study's results, showing significant inhibition of biofilm formation by PA and VRE, are consistent with findings from similar research on AgNPs-functionalized biomaterials. For example, recent work on AgNPs coatings in wound dressings has demonstrated that nanoparticles reduce biofilm formation through both bactericidal action and by interfering with quorum sensing, the communication system bacteria use to regulate biofilm development [124]. Our study corroborates this, particularly with the marked reduction in biofilm formation observed in PA, a pathogen often resistant to conventional treatments.

The more moderate inhibition of biofilm formation by SA observed in this study is also reflected in existing literature, where biofilm-forming gram-positive bacteria and fungi tend to be more resilient to AgNPs treatments. Biofilms formed by these microorganisms are typically more complex and harder to penetrate, which may explain the reduced efficacy observed [125-126]. This suggests that while AgNPs are highly effective against certain pathogens, they may need to be combined with other antimicrobial agents to improve their impact on biofilm-forming gram-positive bacteria and fungal species.

4.2.3. Biocompatibility and cytotoxicity

Biocompatibility is a crucial aspect of any wound healing material, and several studies have evaluated the cytocompatibility of AgNPs at different concentrations [127]. This study's results align with a broad body of literature that demonstrates the biocompatibility of AgNPs when used at controlled concentrations. Cytotoxicity test results confirmed that the concentration of AgNPs in the nanofiber membranes was sufficient to provide antibacterial activity without inducing relevant cytotoxic effects on fibroblast cells.

These findings are consistent with research that emphasizes the importance of balancing AgNPs concentration to avoid cytotoxicity [128]. Studies have shown that silver concentrations above a certain threshold can generate excessive ROS, leading to oxidative stress and cell death [129]. However, at lower concentrations, AgNPs are able to provide effective antimicrobial action while supporting cell viability. In line with these studies, our results demonstrate that the membranes support cell adhesion, proliferation, and viability, with no significant cytotoxic effects observed over the course of testing.

In contrast, other studies have explored the use of alternative materials to mitigate potential cytotoxicity concerns, such as using larger AgNPs, which tend to release fewer ions and may exhibit less cytotoxicity, or combining silver with other materials, such as zinc oxide, to enhance biocompatibility [130-132]. While our study demonstrates that the current concentration of AgNPs does not induce cytotoxicity, future research could explore these alternative strategies to further optimize safety, particularly for long-term applications.

4.2.4. Hydrophilicity and surface modifications

Improving surface hydrophilicity is a common strategy in tissue engineering to enhance cell adhesion and proliferation, moreover increased hydrophilicity is critical for maintaining a moist wound environment, which accelerates the healing process. Similar to other studies that utilize plasma treatments to enhance the hydrophilicity of biomaterials, our results show that the air

plasma treatment significantly improved the wettability of the PCL fibers [133]. This increased hydrophilicity likely contributed to the strong cell adhesion observed in our biocompatibility testing, as hydrophilic surfaces facilitate better cell-material interactions.

In addition, our membranes could absorb and retain water at a level suitable for managing wound exudate, ensuring that the wound remains hydrated without excessive fluid buildup. This balance is crucial in preventing wound dehydration while maintaining a moist environment conducive to tissue regeneration [134].

The use of collagen to further improve biocompatibility also aligns with current literature, where collagen is often used to coat synthetic fibers, providing bioactive sites that promote cell attachment through integrin-mediated interactions [111,113,135]. The positive outcomes seen in our biocompatibility tests mirror those of similar studies, confirming that collagen-coated scaffolds offer a more biologically favorable surface for cell attachment and proliferation.

4.2.5. Implications for wound healing applications

The results from this study suggest that the nanofiber membranes developed using PCL, collagen, and AgNPs have significant potential for use in advanced wound healing applications. The combination of biocompatibility and antimicrobial efficacy makes these membranes suitable for treating chronic wounds, which are often complicated by infection and delayed healing.

The **biocompatibility** of the membranes ensures that they can support tissue regeneration without causing adverse reactions, while the **antibacterial properties** provided by AgNPs can help prevent infection, a major concern in chronic wounds. The ability to **inhibit biofilm formation** is particularly advantageous, as biofilms are often resistant to conventional treatments and contribute to the chronicity of wounds.

However, the limited efficacy of AgNPs against SA highlights the need for further optimization or combination therapies to enhance the spectrum of antimicrobial activity. Exploring alternative antimicrobial agents or combining AgNPs with other agents could help address this limitation and provide broader protection against a wider range of pathogens.

4.3. Clinical implications of the results

The results of this study have several important clinical implications, particularly in the field of advanced wound care. Chronic wounds, such as diabetic ulcers, venous leg ulcers, and pressure sores, present significant treatment challenges due to their prolonged healing times and the frequent occurrence of infections, especially those involving biofilm-forming and antibiotic-resistant bacteria. The multifunctional nanofiber membranes developed in this study, which combine PCL, collagen, and AgNPs, offer promising solutions for these clinical challenges by addressing both the need for structural support and antimicrobial protection.

4.3.1. Enhanced wound healing through structural support and biocompatibility

The biocompatibility of the membranes demonstrated in this study, particularly the high cell viability, indicates that these materials are suitable for promoting tissue regeneration in wounds. The collagen-coated PCL fibers closely mimic the ECM, which is critical for cellular functions such as adhesion, proliferation, and migration. In a clinical setting, this structural similarity to the ECM can enhance the body's natural healing process by providing an environment conducive to fibroblast activity and tissue growth.

Wound dressings that support tissue regeneration are particularly important in treating chronic wounds, where natural healing processes are often impaired. The ability of the nanofiber membranes to support cell proliferation, as shown in the Alamar Blue and MTT assay results, suggests that these membranes could accelerate wound closure by facilitating the growth of

new tissue. This is especially relevant for patients with chronic conditions like diabetes, where wound healing is delayed, and there is an increased risk of wound infections and complications.

Additionally, the hydrophilicity of the membranes, improved through air plasma treatment, enhances fluid absorption and retention, maintaining a moist environment at the wound site. This is a critical factor in clinical wound management, as maintaining the appropriate level of moisture helps promote faster healing and reduces scarring. Clinically, these characteristics could reduce the need for frequent dressing changes, decrease healing time, and ultimately improve patient outcomes.

4.3.2. Antibacterial and biofilm-inhibiting properties for infection control

Infection is one of the primary barriers to effective wound healing, particularly in chronic wounds where biofilm formation can protect bacteria from both the immune system and antibiotics. The results of the antibacterial activity tests demonstrated that the AgNPs - functionalized membranes were highly effective in inhibiting the growth of PA and VRE, both of which are common pathogens in chronic wounds. These results are clinically significant, as antibiotic resistance is a growing problem in wound care, and the ability of AgNPs to target resistant strains like VRE provides an alternative to conventional antibiotic therapies.

The AgNPs also significantly reduced biofilm formation, particularly in PA, one of the most common and difficult-to-treat biofilm-forming bacteria in chronic wounds. The ability of the membranes to inhibit biofilm formation has direct clinical implications for reducing the incidence of chronic wound infections, which are often characterized by persistent biofilms. By preventing biofilm development, the membranes could help overcome a major obstacle to effective wound healing, reducing the risk of infection-related complications and the need for systemic antibiotics.

In clinical practice, preventing infections not only improves patient outcomes but also reduces healthcare costs by minimizing the need for extended hospital stays, surgical interventions to remove infected tissue, and the use of expensive antibiotic treatments. The results of this study suggest that AgNPs-functionalized membranes could serve as an effective antimicrobial wound dressing, reducing the reliance on antibiotics and helping to mitigate the growing issue of antibiotic resistance in healthcare settings.

4.3.3. Addressing antimicrobial resistance

The global rise of antimicrobial resistance (AMR) is a critical concern in clinical settings, particularly in wound care, where infections can significantly delay healing and contribute to severe complications [136]. The membranes developed in this study showed efficacy against resistant strains like VRE, which is a major clinical concern in hospitals and long-term care facilities. By incorporating AgNPs, these membranes offer an alternative to traditional antibiotics, helping to reduce the selection pressure for resistant bacterial strains.

The resistance of SA to AgNPs observed in this study is consistent with clinical challenges, as methicillin-resistant Staphylococcus Aureus (MRSA) continues to pose a significant problem in wound care [137]. However, the membranes' effectiveness against other resistant strains such as VRE highlights their potential role in addressing antimicrobial resistance more broadly. In clinical practice, AgNPs-based wound dressings could be used in conjunction with other antimicrobial agents or therapies to provide a multi-modal approach to infection control, particularly in wounds infected with resistant pathogens.

4.3.4. Potential for broader applications in burn and surgical wounds

The structural and antimicrobial properties of the nanofiber membranes make them well-suited not only for chronic wounds but also for treating acute wounds, such as burns and post-surgical

wounds. Burn wounds are highly susceptible to infection, and the ability of the membranes to prevent bacterial growth and biofilm formation could help reduce the risk of infection in these patients [138]. Additionally, the structural integrity of the membranes, combined with their ability to maintain a moist wound environment, could support the rapid healing of surgical wounds, reducing the risk of post-operative infections and enhancing tissue regeneration.

In the context of burn treatment, the prevention of infection is critical to avoiding complications such as sepsis, which is a leading cause of mortality in burn patients [139]. The AgNPs-functionalized membranes, with their broad-spectrum antibacterial activity and biofilm inhibition, could provide an effective barrier against infection while also supporting the healing of damaged skin.

4.4. Study limitations and considerations for future research

While the results of this study provide promising evidence for the potential use of AgNPs-functionalized nanofiber membranes in wound healing, several limitations must be acknowledged. Addressing these limitations through further research is essential for optimizing the membranes and ensuring their safety and efficacy in clinical applications.

4.4.1. Limited In Vitro focus

One of the primary limitations of this study is its reliance on in vitro testing to evaluate biocompatibility, cytotoxicity, and antimicrobial efficacy. While in vitro models provide valuable insights into cell-material interactions, they do not fully replicate the complexity of the in vivo wound environment, where factors such as immune responses, blood flow, and the presence of multiple cell types influence wound healing outcomes.

In particular, the degradation profile of the nanofiber membranes was assessed in a controlled laboratory setting, but this may differ in vivo, where enzymatic activity, immune responses, and other biological factors could alter the rate of degradation. Future research should include in vivo studies using animal models to better understand the membranes' behavior in a dynamic wound environment. These studies could provide more comprehensive data on degradation rates, biocompatibility, immune response, and overall healing efficacy in the presence of various biological processes.

4.4.2. Pathogen spectrum and resistance issues

While the study demonstrated strong antimicrobial activity against PA and VRE, the results indicated limited efficacy against SA**.** The resistance of SA to AgNPs, as observed in this study, is consistent with findings in the literature that certain bacterial strains can develop mechanisms to evade silver's antimicrobial effects, such as efflux pumps or biofilm-specific phenotypes.

This limitation highlights the need for further optimization of the antimicrobial properties of the membranes. Future research could focus on exploring synergistic effects AgNPs and other antimicrobial agents, such as antibiotics, antifungal agents, or natural antimicrobial peptides, to enhance efficacy across a broader spectrum of pathogens. Additionally, varying the size, shape, and surface functionalization of AgNPs could be investigated to overcome bacterial resistance and improve the overall effectiveness of the membranes.

4.4.3. Potential for silver toxicity

Although the cytotoxicity assays demonstrated that the AgNPs concentration used in this study did not induce significant toxicity to fibroblast cells, silver toxicity remains a concern, particularly with long-term exposure. AgNPs can generate ROS, which, at high concentrations, may lead to oxidative stress and cell damage in human tissues. The balance between

antimicrobial efficacy and cytocompatibility is delicate, and silver's long-term effects on wound healing and surrounding healthy tissue have yet to be fully explored.

Future research should focus on optimizing the concentration and release rate of AgNPs to maintain antimicrobial efficacy while minimizing potential toxicity. Controlled-release systems, where silver nanoparticles are encapsulated within a biodegradable material, could be developed to regulate the release of silver ions over time, preventing excessive accumulation and reducing the risk of cytotoxicity. Additionally, long-term in vivo studies should be conducted to evaluate the potential cumulative effects of silver exposure and ensure the membranes are safe for prolonged use in clinical settings.

4.4.4. Limited focus on bioactive molecule delivery

While the membranes' ability to promote proliferation was confirmed, the study did not explore the potential for these scaffolds to serve as vehicles for bioactive molecule delivery, such as growth factors or therapeutic drugs. The incorporation of bioactive molecules could significantly enhance the wound healing process by stimulating specific cellular functions, accelerating tissue regeneration, or modulating the immune response.

Future research should investigate the feasibility of loading the nanofiber membranes with bioactive agents. Growth factors such as vascular endothelial growth factor (VEGF) or platelet-derived growth factor (PDGF) could be incorporated into the membranes to promote angiogenesis and tissue formation. Additionally, the development of controlled-release systems within the membranes could enable the sustained delivery of these agents, providing a multifunctional scaffold that not only supports structural tissue regeneration but also actively promotes healing at the molecular level.

4.4.5. Biofilm resistance and long-term antimicrobial efficacy

The study demonstrated the ability of the AgNPs-functionalized membranes to inhibit biofilm formation, particularly for PA and VRE. However, the long-term efficacy of the antimicrobial action was not fully explored. Over time, bacteria can adapt and develop resistance to antimicrobial agents, and biofilms may still form if the antimicrobial efficacy decreases.

Future studies should investigate the long-term antimicrobial performance of the membranes, including their sustained ability to prevent biofilm formation over extended periods. Testing under chronic wound models could provide insights into how the membranes perform over time in real-world scenarios. Additionally, combining AgNPs with other anti-biofilm agents or coatings that disrupt biofilm structure could improve the membranes' long-term efficacy.

4.5. Potential applications of the new dressing concept

The multifunctional nanofiber membranes, which combine PCL, collagen, and AgNPs, offer significant potential for various clinical applications, particularly in wound care. The membranes address key challenges in wound healing, including promoting tissue regeneration, preventing infection, and controlling biofilm formation. These properties make them suitable for both chronic and acute wound management in different patient populations and clinical settings.

In chronic wound management, these membranes could be particularly valuable for treating conditions such as diabetic ulcers, venous leg ulcers, and pressure sores. Chronic wounds often have prolonged healing times and are prone to infection. The membranes developed in this study, with their antimicrobial properties derived from AgNPs, can effectively prevent bacterial infections, including those caused by drug-resistant strains like PA and VRE**.** This infection control capability is crucial, as infections significantly delay wound healing and can lead to

serious complications. Moreover, the membranes' ability to inhibit biofilm formation, especially against PA, addresses a key barrier in the treatment of chronic wounds, where biofilms protect bacteria from antibiotics and immune responses. By preventing biofilm development, the membranes may reduce infection-related complications and improve healing outcomes.

Another important feature of these membranes is their capacity to maintain moisture at the wound site. The enhanced hydrophilicity achieved through plasma treatment allows the membranes to absorb excess exudate while keeping the wound environment moist, which is essential for promoting tissue regeneration and reducing scarring. This characteristic is beneficial in chronic wound care, where maintaining the right moisture balance can accelerate healing and decrease the frequency of dressing changes, improving patient comfort.

In acute wound care, including surgical and burn wounds, the membranes provide both antimicrobial protection and mechanical support, making them suitable for post-surgical wound management and the treatment of traumatic injuries. After surgery, reducing the risk of infection and promoting rapid tissue healing are critical. These membranes can be applied immediately following surgical procedures to protect the wound site, minimize infection risk, and support healing without the need for frequent dressing changes. Similarly, in burn treatment, where the skin's protective barrier is compromised, the antimicrobial properties of AgNPs and the ability to prevent biofilm formation would help lower the risk of infection, a common complication in burn wounds. The structural integrity of the membranes also supports the healing of damaged tissue, helping to close wounds more effectively.

Diabetic foot ulcers are another area where the membranes could have a significant impact. These ulcers are difficult to treat due to the increased risk of infection, poor blood circulation, and delayed healing often seen in diabetic patients. The membranes' ability to inhibit infections

caused by common diabetic wound pathogens could reduce the need for systemic antibiotics and help prevent complications. Additionally, the collagen-coated PCL fibers mimic the natural ECM, promoting fibroblast adhesion and tissue regeneration, which are essential for closing diabetic wounds. In the long term, this could lead to fewer amputations and improved quality of life for diabetic patients.

The membranes also have potential in military and emergency medicine, where rapid wound treatment and infection control are essential. Their immediate antimicrobial protection and mechanical strength make them ideal for use as field dressings in contaminated environments. In battlefield scenarios or emergency situations, these membranes could be applied quickly to stabilize wounds, prevent infection, and provide structural support during transportation to medical facilities. Their use in such contexts could significantly reduce the risk of infection and subsequent complications, especially when immediate surgical intervention is not feasible.

In addition to their current wound care applications, the membranes offer significant potential for future development as drug delivery platforms. By incorporating bioactive molecules such as growth factors or anti-inflammatory agents, the membranes could be adapted to accelerate healing in wounds that require more complex interventions. For instance, delivering growth factors like vascular endothelial growth factor (VEGF) or platelet-derived growth factor (PDGF) could enhance angiogenesis and tissue regeneration in large wounds or those with poor vascularization, such as diabetic ulcers or burn injuries. Similarly, the controlled release of anti-inflammatory agents from the membranes could help manage inflammation in chronic wounds, creating a more favorable environment for tissue repair.

The versatility and multifunctionality of these membranes make them suitable for a wide range of clinical applications. By providing antimicrobial protection, promoting tissue regeneration, and offering structural support, the membranes represent a promising tool for addressing the

challenges of wound care in both chronic and acute settings. As the technology continues to develop, the potential for integrating additional therapeutic functions, such as drug delivery, could expand the applications of this dressing concept even further, improving outcomes for patients with complex wounds and reducing the burden of wound care on healthcare systems.

5. CONCLUSION

5.1. Summary of key findings

This doctorate thesis investigated the development and characterization of multifunctional nanofiber membranes incorporating PCL, collagen, and AgNPs for wound healing applications. The primary findings demonstrated that these membranes exhibit a unique combination of structural support, biocompatibility, and antimicrobial properties.

The **morphological analysis** confirmed that the electrospun nanofiber membranes had a highly porous structure with fibers resembling the ECM, which is crucial for cell attachment and proliferation. **Mechanical testing** revealed that the membranes possess adequate tensile strength and flexibility, making them suitable for conforming to wound sites while providing mechanical support. **Biocompatibility testing**, showed high levels of cell viability and proliferation on the membranes, particularly those functionalized with collagen, indicating that the scaffold is conducive to fibroblast adhesion and growth.

The **antibacterial activity** of the membranes was also confirmed, with AgNPs effectively inhibiting the growth of **Pseudomonas Aeruginosa** and **Vancomycin-resistant Enterococcus**, and significantly reducing biofilm formation. However, limited efficacy against **Staphylococcus Aureus** suggests the need for further optimization. Importantly, the AgNPs did not exhibit cytotoxic effects at the concentrations used, ensuring that the membranes can provide antimicrobial protection without compromising cell health.

5.2. Contribution of the research to the field of Plastic Surgery and Wound Healing

This research contributes to the growing body of knowledge in the fields of plastic surgery and wound healing by presenting a novel wound dressing concept that integrates multiple functions: structural support, infection prevention, and tissue regeneration.

In plastic surgery, the ability to promote tissue regeneration while preventing infections is critical, particularly in procedures where wound healing may be complicated by infection or compromised tissue health. The development of nanofiber membranes that mimic the natural ECM and offer antimicrobial protection addresses key challenges in post-operative wound management. For example, these membranes could be used in reconstructive surgeries or skin grafting procedures, where protecting the wound site from infection is essential for successful healing.

In the broader context of wound healing, particularly for chronic wounds such as diabetic ulcers or pressure sores, the membranes developed in this research provide a dual advantage. By supporting tissue regeneration through collagen-coated PCL fibers and preventing infection with AgNPs, the membranes have the potential to improve healing outcomes, reduce the frequency of wound complications, and lower the dependency on systemic antibiotics. The inhibition of biofilm formation, a major issue in chronic wounds, highlights the membranes' capacity to address one of the most persistent barriers to effective wound care.

5.3. Suggestions for future research

While this study demonstrates significant promise for the use of multifunctional nanofiber membranes in wound healing, several avenues for future research should be pursued to further optimize the technology and expand its applications:

1. **In vivo testing**: to translate the findings of this study into clinical applications, comprehensive in vivo studies are needed. Testing the membranes in animal models or clinical trials would provide valuable insights into their behavior in real wound environments, including their degradation rate, interaction with host tissues, and overall efficacy in promoting wound closure and preventing infection.
2. **Optimization of antimicrobial properties**: the limited efficacy of the AgNPs against Staphylococcus aureus suggests that further research is needed to optimize the antimicrobial properties of the membranes. Investigating the use of alternative antimicrobial agents or combinations of silver with other antibiotics, antimicrobial peptides, or natural extracts could enhance the membranes' effectiveness against a wider range of pathogens, including resistant bacterial strains and fungi.
3. **Controlled release systems**: future studies could explore incorporating controlled-release systems for both antimicrobial agents and bioactive molecules, such as growth factors or anti-inflammatory drugs. Controlled delivery of bioactive agents would allow for sustained therapeutic effects over the course of healing, potentially accelerating tissue regeneration and further improving wound outcomes.
4. **Application in specialized wound care**: the membranes' applicability to specific types of wounds, such as burn wounds, surgical incisions, and diabetic ulcers, should be explored in greater detail. Each wound type presents unique challenges, and understanding how the membranes perform under different clinical conditions will help refine their use in diverse medical contexts.

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7. FIGURES