UNIVERSIDADE D
COIMBRA

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THERAPEUTIC EFFECTS OF NOVEL LL37-AUNPS COATED DRESSING FOR THE ENHANCED WOUND HEALING

Dissertação no âmbito do Mestrado de Biotecnologia Farmacêutica, orientada pelo Doutor Akhilesh Rai e pelo Professor Doutor João Nuno Moreira e apresentada à Faculdade de Farmácia da Universidade de Coimbra

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"*If we knew what it was we were doing, it would not be called research, would it ?"*

-Albert Einstein

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Acknowledgments

Firstly, I would like to show my gratitude for Dr. Akhilesh Rai and Dr. Lino Ferreira, for allowing me to do my thesis project at their group. I got to learn immensely in the past year in their labs. I had the opportunity to give a speech about Cell Culture at the "VII AEICBAS Biomedical Congress", and to supervise 4 MSc and 1 BSc Students. Specifically, I provided them with technical training and in outlining their internship report.

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ABSTRACT

Despite increasing awareness of the pathophysiologic events that take place in the development of wounds of various underlying etiologies, impaired wound healing and its medical complications remain one of the most prevalent and economically burdensome healthcare issues in the world. Approximately 6.5 million US patients are affected by chronic wounds and more than US$25 billion are spent annually for the treatment of this condition. Chronic wounds have many psychosocial consequences, ranging from social isolation to mental malaise. In addition, due to an increase in the aging population and, a rise in diabetes and obesity incidence around the world, impaired wound healing will continue to increase. Given this, research related to tissue regeneration in chronic wound repair is vital, as well as the development of new therapeutics is absolutely necessary and important to satisfy the unmet clinical need.

In this thesis, we studied the therapeutic effects of LL37-gold nanoparticles coated polyurethane (LL37-AuNP-PU) dressing, which is a novel technology developed by our lab. We performed biocompatibility assays against the human keratinocytes cells (HaCaT cells) and showed that the viability of HaCaT cells was not compromised, indicating that LL37-AuNP-PU dressing is not toxic to the cells. In addition, we also showed that this treatment induces wound closure through the expression of more keratins in the proliferative edges of the wound and in the wound gap. Furthermore, LL37-AuNP-PU dressing regulates the activation of macrophages and improves the progression of the inflammatory stages of wound healing, which makes this environment more prone to the beginning of proliferative and remodeling stages of wound healing.

Overall, the results presented in this thesis demonstrated that LL-37-AuNP-PU dressings could potentially be used for the treatment of chronic wounds especially the diabetic wounds while preventing bacterial infection.

Key Words: LL37-AuNP-PU dressing; Chronic Wounds; Macrophage regulation; Keratinocytes; Treatment

RESUMO

Apesar da crescente consciencialização dos eventos fisiopatológicos que ocorrem no desenvolvimento de feridas de várias etiologias, a ineficiente cicatrização e as complicações médicas associadas continuam a ser um dos problemas de saúde mais prevalentes e economicamente onerosos no mundo. Aproximadamente 6.5 milhões de doentes dos Estados Unidos são afetados por feridas crónicas e mais de 25 mil milhões de dólares são gastos anualmente no tratamento desta condição. As feridas crónicas têm muitas consequências psicossociais, que vão desde o isolamento social ao mal-estar mental. Além disso, devido ao aumento da população idosa no mundo e a um aumento na incidência de diabetes e obesidade, a ineficaz cicatrização de feridas irá continuar a aumentar. Dado isto, a investigação de regeneração de tecidos nas feridas crónicas tornou-se vital, assim como o desenvolvimento de novas terapêuticas que se tornou absolutamente necessário e importante para satisfazer as necessidades clínicas existentes.

Nesta tese, estudámos os efeitos terapêuticos do *patch* de poliuretano revestido de nanopartículas de ouro com o péptido LL37 (LL37)-AuNP-PU, uma nova tecnologia desenvolvida pelo nosso laboratório. Realizámos ensaios de biocompatibilidade e demonstrámos que a viabilidade de queratinócitos humanos não foi comprometida, indicando que o *patch* LL37-AuNP-PU não é tóxico para as células humanas. Em adição, também demonstrámos que este tratamento induz a cicatrização da ferida através da expressão de mais queratina nas extremidades proliferativas da ferida, assim como na ferida. Por conseguinte, o LL37-AuNP-PU regula a ativação de macrófagos e melhora a resposta inflamatória, assim como a sua progressão no processo de cicatrização de feridas, o que torna esse ambiente mais propício ao início dos estágios proliferativos e de remodelação da cicatrização.

No geral, os resultados apresentados nesta tese demonstraram que o *patch* LL-37-AuNP-PU pode ser potencialmente utilizado para o tratamento de feridas crónicas, especialmente feridas diabética, ao mesmo tempo que previne infeções bacterianas.

Palavras-Chave: Patch LL37-AuNP-PU; Feridas crónicas; Regulação de macrófagos; Queratinócitos; Tratamento

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to the nucleus of cells. The red and green stained cells represent M1 and M2 macrophages respectively.

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LIST OF ABBREVIATIONS

- AMP: Antimicrobial Peptides
- Bcl-XL: B-cell lymphoma-extra large
- CAMP: Cathelicidin Antimicrobial Peptide
- cAMP: Cyclic Adenosine Monophosphate
- ECM: Extracellular Matrix
- EGF: Endothelial Growth Factor
- EGFR: Epidermal Growth Factor Receptor
- FDA: Food and Drug Administration
- FGF: Fibroblast Growth Factor
- FPRL-I: Formyl Peptide Receptor Like-1
- FPR2: Formyl Peptide Receptor 2
- KGF: Keratinocyte Growth Factor
- HaCaT cells: Human Keratinocytes Cells
- IFN-γ: Interferon-gamma
- IL-1: Interleukin 1
- IL-1β: Interleukin 1 beta
- IL-6: Interleukin 6
- IL-10: Interleukin 10
- IL-19: Interleukin 19
- IL-1Ra: Interleukin-1 Receptor Antagonist
- KFG: Keratinocyte Growth Factor
- LPS: Lipopolysaccharide
- LL37-AuNP: LL37 conjugated Gold Nanoparticle
- LL37-AuNP-PU: LL37 conjugated Gold Nanoparticles-Polyurethane
- MMP: Matrix Metalloproteinases
- NET's: Neutrophil Extracellular Traps
- NO: Nitric Oxid
- NP: Nanoparticle
- PDGF: Platelet-derived Growth Factor
- P2X7: P2X purinoceptor 7

- ROS: Reactive Oxygen Species
- TGF-α: Transforming Growth Factor-alpha
- TGF-β: Transforming Growth Factor beta
- VEGF: Vascular Endothelial Growth Factor

INTRODUCTION

INTRODUCTION

Wound healing

Despite increasing awareness of the pathophysiologic events that take place in the development of wounds of various underlying etiologies, impaired wound healing and its medical complications remain one of the most prevalent and economically burdensome healthcare issues in the world. The market of annual wound care products has reached US$ 15.3 billion by 2010 and the need for post-surgical wound care is sharply on the rise. In the US alone, more than 100 million acute wounds reported annually, including surgical incisions, trauma, and burns, while chronic wounds affect 6.5 million US patients, being considered the "new global epidemic" [1]. More than US$ 25 billion is spent annually on the treatment of non-healing wounds and the burden is growing rapidly due to an increase in health care costs, an aging population and a sharp rise in the incidence of diabetes and obesity worldwide [2]. Recent data indicate that 11.7 % of the Portuguese population is diabetic [3].

One of the most serious and debilitating complications of diabetes is precisely the development of chronic non-healing wounds. More importantly, diabetes is the leading cause of nontraumatic leg amputations in the US alone. Besides the increase in diabetic ulcers, pressure ulcers in critical care and intensive care patients are also increasing. Chronic wounds have many psychosocial consequences, including stress, pain and social isolation [4]. Current treatment options are limited, costly and inefficient. Considering the increase in the incidence of diabetes and obesity, the investigation into tissue regeneration in chronic wound repair is vital, and the development of new therapeutics to satisfy the unmet clinical problems is needed.

What are they?

According to the Wound Healing Society (WHS), a wound can be described as a defect or break in the skin due to physical damage, thermal damage and medical or physiological conditions. In a resume, a wound is a result of "*disruption of normal anatomic structure and function"* of the skin.

A wound can be classified as acute or chronic based on the nature of their healing process. An acute wound has a normal healing process, meaning that the damaged tissue is able to heal completely with minimal scarring within the expected time frame, which is usually 8 to 12 weeks. Typically, an acute wound is caused by mechanical injuries due to external factors and those include: abrasions, that can be caused by frictional contact between the skin and harsh surfaces; penetrating wounds, caused by sharp materials, like knives; surgical wounds, caused

by surgical incisions; chemical wounds, that can be caused by exposure to radiation, electricity, corrosive chemicals and thermal burns. On the other hand, chronic wounds have a slow healing process and take more than 12 weeks to heal along with a tendency to relapse. These wounds become chronic due to the failure of events that take part in the healing process, because of persistent tissue damage that is often the result of prolonged pathogenic inflammation, re-occurring infections caused by pathogenic bacteria or due to diseases such as diabetes (e.g.: foot ulcers). Wounds can also become chronic if the primary treatment was done poorly. Additionally, other lifestyle-related factors such as diet and smoking contribute to the development of chronic wounds.

Besides the acute and chronic classification, wounds can also be classified, according to the number of skin layers affected, into (1) superficial wound, a wound that only affects the outermost layer of the skin, which is the epidermal layer; (2) partial full-thickness wound, that affect both the epidermal layer and deeper skin layers (including blood vessels, hair follicles or sweat glands); and (3) full-thickness wound, when the epidermis, dermal layers and the underlying subcutaneous fat (or deeper tissues) are damage all at once [5,6,7]

Wound Healing Mechanism

Wound healing is a well-orchestrated biological process that occurs through a line of consecutive biological events that has a final goal of healing the injury and repairing the damaged tissue with minimal scarring [8]. The healing process can be divided into four stages, regardless of acute or chronic wounds: (1) hemostasis; (2) inflammation; (3) proliferation and; (4) tissue remodeling [6, 7]. This event is only triggered when the natural skin barrier is compromised due to an injury caused by trauma. Moreover, the normal function and anatomical structure of the skin are maintained due to the interaction between T-Cells, resident keratinocytes, mast cells, fibroblasts, macrophages, and dendritic cells. Importantly, the symbiotic relationship between the skin microorganisms and the host protects our skin from more harmful or pathogenic microorganisms. As an example, *Staphylococcus epidermidis* can secrete antibacterial compounds and induce the expression of antimicrobial peptides (AMP) or, on the contrary, induce the reduction of inflammation [29].

Hemostasis [seconds to minutes after tissue injury]

Upon injury, the immediate response of the body is to prevent blood loss (exsanguination) and stimulate hemostasis by contracting the smooth muscle in the blood

vessels, constricting the arterial vessels through the increase of cytoplasmic calcium levels [6]. This stage is activated as soon as a disruption of the skin integrity occurs and leads to the decrease of blood flow in the wound area. Hemostasis causes tissue hypoxia, which consequently promotes the release of neurotransmitters and hormonal molecules, further inducing the production of nitric oxide (NO), cyclic adenosine monophosphate (cAMP) and other vasoactive metabolites. The secretion of these molecules stimulates the contraction/relaxation of the smooth muscle through the activation of signaling pathways [6,9]. As the level of calcium increases in the wound to promote smooth muscle contraction, histamine is also being released from the activated mast cells in the damaged tissue to promote the increase of the membrane permeability, leading to the passage of inflammatory cytokines.

In addition to the vessel constriction mechanism, further blood loss is prevented through the activation of the coagulation cascade using three major mechanisms: contact activation cascade (intrinsic pathway), tissue factor pathway (extrinsic pathway) and platelet activation [8].

The exposure of subendothelial collagen and the formation of thrombin lead to the activation of platelets located in the intravascular space. Activated platelets are able to initiate the coagulation cascade, leading to the formation of a fibrin clot. This clot protects the denuded wound and serves as a provisional scaffold for other cells that can migrate during the repair process. Importantly, the clot also serves as a reservoir of cytokines, growth factors vasoactive substances such as platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF-β), fibroblast growth factor (FGF), endothelial growth factor (EGF), serotonin, thromboxane A2, platelet factor IV, prostaglandins and histamine, which are released from the activated platelets degranulate [11]. These factors can initiate the early events of the wound closure process, providing chemotactic cues to recruit circulating inflammatory cells to the wound site. Additionally, these factors promote the re-epithelialization and connective tissue contraction and, stimulate the characteristic wound angiogenic response [10,11].

Inflammation [2 hours – 2 to 6 days after injury]

After hemostasis, the activated cells and pathways generate an innate immune response that leads to the initiation of the inflammatory stage of wound healing. The main goal of the inflammation stage is to decontaminate the wound site of any harmful bacteria or other invaded microorganisms that could potentially cause infection [7]. In order to eliminate microorganisms, neutrophils (the polymorphonuclear leukocytes) are the first cells to arrive

at the wound site. Additionally, an immediate expression of AMP, such as human cathelicidin LL-37 is induced at the wound site [7]. The neutrophils enter the wound bed after 1h and are predominantly present for the first 72 h after the injury. The neutrophil levels reach their peak at approximately 48 h after injury [8]. Neutrophils eliminate microorganisms through different mechanisms such as (1) phagocytosis; (2) degranulation of neutrophils; and (3) neutrophil extracellular traps (NET's).

Phagocytosis is a process in which the neutrophils directly ingest and destroy the foreign body [6]. The receptors on the surface of the neutrophils recognize the foreign body and surround the target through a process called pseudopodia. Afterward, the neutrophil (with the target inside the cell) fuses with lysosomes, leading to the acidification of the phagosome and, consequently the elimination of the target [13].

Neutrophils go through a differentiation period in the bone marrow until they reach their final form. During this time, neutrophils accumulate granules and pack them with antimicrobial molecules and proteases [12]. Upon signaling, neutrophils release the toxic content of the cytoplasmic granules, such as lactoferrin, proteases, neutrophil elastase and cathepsin by a process called degranulation [6]. The secreted molecules have the main function of eliminating the bacteria and dead host cells tissues, to facilitate cell migration of inflammatory cells to the wound bed [12]. Additionally, the released proteases from neutrophils remove the damaged components of the extracellular matrix (ECM).

As an alternative death mechanism, NET's release its own chromatin fibers coated with antimicrobial proteases, which trap and eliminate bacteria. This method is efficient even upon neutrophil death [14].

After completing their function of cleaning and decontaminating the wound, neutrophils enter into an apoptotic mode. Subsequently, activated monocytes are differentiated to macrophages and recruited at the injured sites to help in host defense [15]. Macrophages engulf the dead neutrophils through the phagocytosis mechanism [6,8,16]. Besides removing the dead neutrophils, macrophages also continue the decontamination of the wounded tissues by synthesizing NO [16]. Importantly, macrophages also play a crucial role in the modulation of the inflammatory response. Macrophages have a large storage of growth factors like TGF-β and EGF that are essential for the stimulation of angiogenesis and formation of granulation tissue, which is the result of the combination of activated fibroblast and macrophage stimuli [6,15].

In the later stage of the inflammatory response, it has been shown that macrophages change from a pro-inflammatory phenotype to an anti-inflammatory phenotype (alternatively activated macrophage) due to the release of anti-inflammatory cytokines and other growth

factors. This transition occurs if the wound is completely cleared from any harmful microorganisms [6,7].

Proliferation (From day 4 to day 14)

The proliferation stage of wound healing begins as soon as hemostasis is reached, inflammation is controlled and the wound bed and surrounding area are free from debris that could lead to a prolonged inflammation stage. This stage is characterized by angiogenesis, the formation of granulation tissue, collagen deposition, re-epithelization and, at last, wound retraction.

Angiogenesis is essential for the formation of new blood vessels at the wound site, in order to provide oxygen and nutrients to the new tissue and for the closure of the wound by contraction and fibroplasia [16,17]. This process is triggered right after the formation of the clot plug. The activated platelets are stimulated to release TGF-β, PDGF, FGF. In addition, at this stage of wound healing, fibroblast and endothelial cells are the most proliferating cells found in the wound bed [6,16]. The release of vascular endothelial growth factor (VEGF) from keratinocytes and the release of FGF by local fibroblasts promote the proliferation of endothelial cells in the wounded area. In return, new capillary blood vessels are formed [6,8,16].

The granulation tissue formation begins three to four days post-injury. This stage is characterized by the proliferation of fibroblasts to the wounded area, which becomes the predominant type of cells in the wound bed. The proliferation of fibroblasts is promoted by FGF, TGF-β1, PDGF which are released from the activated macrophages. Once the migration of fibroblasts to the wound bed occurs, they produce new ECM proteins (hyaluronan, fibronectins, and proteoglycans), synthesize collagen (predominantly type III) and fibronectin. Once sufficient ECM is produced, fibroblasts transdifferentiate to myofibroblasts (induced by TGF-β1) and develop pseudopodia to connect with surrounded healthy cells and promote further wound contraction [6,8,16].

The re-epithelialization occurs when healthy keratinocytes, located at the surrounding area of the wound, start to proliferate at the border of the damaged tissue to produce a protective barrier to prevent fluid losses and bacterial invasion and to aid in wound contraction. Activated platelets and macrophages release EGF and transforming growth factor-alpha (TGF-α), which leads to keratinocyte migration and proliferation. In turn, fibroblasts secrete keratinocyte growth factor (KFG) and interleukin 6 (IL-6), which stimulates further keratinocyte migration and proliferation. Keratinocyte mobility is also facilitated by the

expression of surface integrin receptors, which will connect to several components of the new ECM (e.g.: stromal type I collagen, fibronectin, etc.). However, overproduction increases the risk of hypertrophic scars [6,8,16].

Wound retraction is initiated approximately 7 days after the injury. This process is mediated by myofibroblasts that contain α-smooth muscle actin fibrin. The interaction between the actin and myosin promotes the contraction of wound edges, leading to wound closure [6,8,16].

The final stage of the wound healing is the tissue remodeling, which consists of a massive change in the temporary ECM formed previously, in order to form new mature and normal epithelium of the scar tissue. This phase is characterized by the reorganization, degradation, and resynthesize of new ECM. Once the keratinocytes form a monolayer, their migration to the wound site is inhibited and a new stratified epidermis is formed. In parallel, a subjacent basal lamina is re-established from the edges of the wound to the center. During tissue remodeling, type III collagen begins to be degraded and replaced with type I collagen. In addition, the production of fibronectin and hyaluronan is reduced and degraded by matrix metalloproteinases (MMPs), which are produced by fibroblasts, keratinocytes, and macrophages. In turn, collagen fibers become thicker and stronger, increasing the tensile strength of the tissue. During this process, the majority of blood vessels, inflammatory cells and fibroblasts are degraded through apoptosis and other cell death mechanisms. However, wounds never regain 100% of the tensile tissue strength, since the number of cells in the previously damaged tissue area is reduced [6,8,16].

Role of LL37 AMP in Wound Healing

Wound healing is a complex biological process. Multicellular organisms have evolved to carry host defense molecules like AMP, also known as *"Natural Antibiotics"*, that are part of the human cathelicidin family. Cathelicidin plays an important role in the innate immune defense system against invasive microbial infections. AMPs are the first line of defense against pathogens and their goal is to control the microbial proliferation and to modulate the immune response of the host to a broad spectrum of physical injuries or damages.

AMPs play an important role both in normal skin function and in various unhealthy skin conditions. AMP was first observed in mammalian skin when cathelicidin PR-39 was discovered in porcine wound fluid. At that moment, human LL37 was observed in epidermal keratinocytes. In general, mainly defensins and cathelicidin (LL37) are secreted by various epithelia in the skin, not only, to serve as antimicrobial agents and contribute to innate immunity, but to stimulate

and regulate inflammation, angiogenesis and wound healing [17]. They are also produced by monocytes, macrophages, NK cells, neutrophils, mast cells, B cells, and epithelial cells.

LL37 AMP is encoded by the gene CAMP, and it is a small cationic peptide (~4.5 kDa), with an α-helical structure and amphipathic characteristic [18]. The gene expression of hCAP18/LL37 can be up-regulated by several pro-inflammatory cytokines, growth factors, the active form of vitamin D and bacterial (*Gram*-positive and *Gram*-negative bacterias) toxins released upon infection at inflammation sites [18].

It is well-known that LL37 has broad antimicrobial activity against bacterias, fungi, and enveloped viruses. The mechanism in which they act is basically through the disruption of the cell membrane of the invading microbes by promoting the formation of pores, which will eventually lead to cell death. The exact killing mechanism of AMP to the invading pathogens is still unsure, however, three concepts such as: **"The Carpet Model"**, **"The Barel-Stave Model"** and, **"The Toroidal Pore Model"** have been proposed [18,19].

In the "The Carpet Model" the peptides aligned in parallel to the cell membrane without intercalating with it. A saturation point is reached that results in extensive wormhole formation, causing the abrupt lysis of the microbial cells. In the "The Barel-Stave Model" the peptides are positioned perpendicularly to the cell membrane of the invasive pathogen, creating a sort of "barrel" type format, in which the peptides assume a "stave" characteristic. The non-polar side chains associate with the hydrophobic fatty acid tails at the inside of the phospholipid bilayer, and the hydrophilic side-chains are oriented inward into the water-filled pore. At last, in "The Toroidal Pore Model" peptides are positioned perpendicularly to the cell membrane, and form pores in which the hydrophilic components interact with the residues of the phospholipids, and the hydrophobic part of the AMPs interacts with the membrane lipids.

In addition to the broad spectrum of antimicrobial activity of AMPs, they are able to inhibit/neutralize the pro-inflammatory activity of LPS, and therefore modulate the inflammatory response. LL37 has a chemoattractant function towards neutrophils, monocytes, macrophages, eosinophils and mast cells to the site of infection or to the wound area via Formyl Peptide Receptor Like-1(FPRL-1). Besides that, they have also the ability to promote re-epithelization upon injury and accelerate wound closure by promoting the proliferation and migration of keratinocytes and epithelial cells via the transactivation of epidermal growth factor receptor (EGFR) [18,19].

LL37 peptides promote the increase of the inflammatory response by increasing the lifespan of neutrophils. Nevertheless, LL37 has anti-inflammatory properties, since they promote the synthesis and release of anti-inflammatory cytokines like interleukins 10 (IL-10), 19 (IL-19) and interleukin-I receptor antagonist (IL-1Ra) by stimulating macrophages, keratinocytes, and macrophages. Besides that, they are able to inhibit the production of TNF-α and NO and decrease the level of reactive oxygen species (ROS) in a non-significant manner during polarization of M1 macrophage by LPS and interferon-gamma (IFN-γ). In addition, they can decrease the production of TNF-α in M2 macrophage polarized by LPS [18,19].

Apart from the chemotactic effect and modulation of cytokine release, LL37 regulates apoptosis of cells involved in infection, promotes angiogenesis and enhances wound healing. LL37 is able to suppress apoptosis of epithelial cells and neutrophils, thereby prolonging the production of chemokines and cytokines and the clearance of pathogens at the site of infection. This suppression is realized through the action of LL37 on the FPR2 and P2X purinoceptor 7 (P2X7) receptor with a mechanism that involves ERK1/2 activation leading to a decreased caspase-3 activity and an increased B-cell lymphoma-extra large (bcl-xl) anti-apoptotic protein expression [20]. The formation of new blood vessels is a prerequisite of tissue repair and wound healing. Angiogenic properties of LL37 have been associated with binding via FPRL1, a G protein-coupled found in endothelial cells [21]. Moreover, LL37 is able to recruit endothelial progenitor cells to the site of wound healing and induces their proliferation through the activation of the NFjB dependent pathway [22].

Furthermore, the concentration of LL37 and the duration of the stimulus further affect the response. Due to their wide range of properties, many groups have recognized the therapeutic potential of exogenous LL37 in several applications such as novel antibiotics, anti-HIV drugs, anti-cancer drugs and chronic treatments [23]. A Swedish company (Pergamum AB) has reported recently the results of phase I/ II clinical trials [24]. The company announced that the primary safety and tolerability end-point for LL37 was met for patients with venous leg ulcers. The results further show that patients treated with LL37 (twice per week; 0.5 mg/mL) had a statistically significant improved healing rate compared with placebo. However, the peptide was administered twice per week and new systems for the sustained release of the peptide while potentiating its activity may be needed.

Therefore, to translate LL37 based therapeutics in the market, there is a need to simplify the structure (in order to preserve helicity and hydrophobic nature) and reduce host-cell toxicity whilst maintaining stability to general proteolysis. One possible solution is represented in the use of nanoparticles (NPs). The NPs present a high density of AMPs per

surface area and therefore activate and prolong the regenerative mechanism. NPs reduce the degradation of the peptide by proteases in the wound. The chemical immobilization of LL37 on nanoparticles (NP) permits the tune of the physiochemical properties, notably amphipathicity, helicity and charge of LL37. It may enhance LL37 activity by prolonging the activation of receptors by perturbing more efficiently the fluidity of the cell membrane due to the cooperative effect of LL37 peptides attached covalently to the surface of the NP.

Below is a summarize figure (**Figure 1**), illustrating the biologic activity of LL37 expressed in wound condition [18]:

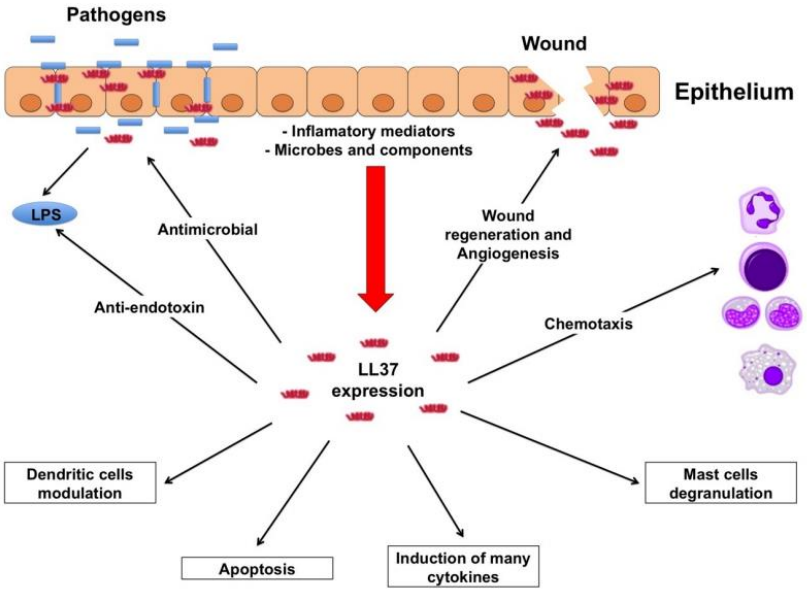


Figure 1.- Biological Activity of LL37 AMP [18]

Clinical treatments of wounds:

Currently, chronic and acute wounds of different etiologies are treated using a multistep approach based on contemporary knowledge of wound healing and known by the acronym **TIME**. First, nonviable tissues (**T**) from within and around a wound are removed using surgical debridement or debriding agents, such as bacterial collagenase. Second, infection and inflammation (**I**) are minimized with antibiotics and anti-inflammatory preparations. Third, moisture (**M**) imbalance is corrected, generally with carefully selected dressings. Last step, epithelialization (**E**) and granulation tissue formation are promoted by the application of specific therapies, such as cell-based therapies or growth factors [25]. The use of **TIME** and current therapies are not always effective in some wounds. In fact, the success of a therapy

depends on the detailed understanding of the molecular and cellular components present in each wound bed. Recent advances in understanding those components using novel diagnostic approaches as for example the "barcoding", can lead to personalized diagnosis and therapies and therefore better therapeutic outcomes [26]. Depending on the type of wounds, current treatments consist of specific dressings, skin substitutes, and growth factors. There is a variety of wound dressings in the market, all of which function to preserve hydration within the wound in order to optimize regeneration, warm, protect against infection, and avoid disruption of the wound base. The most currently available bioactive wound dressings are made using chitosan as "HemCon® Bandage", hyaluronic acid, collagen and silicon as the product "Biobrane" [27]. In addition, other biomaterials that are currently being investigated for wound dressings consist of alginates, heparin, cellulose, and gelatin [28]. Being these materials biodegradable and part of the natural tissue matrix, they play an active part in the new tissue formation.

Dressings cannot replace lost tissue, particularly missing dermis as occurs in chronic wounds. For the treatment of these cases, bioengineered skin substitutes and growth factors represent the only Food and Drug Administration (FDA) approved products nowadays. Skin substitutes consist of an autograft, allograft or xenograft. Bioengineered skin substitutes such as polymers, naturals or synthetic act as scaffolds for tissue-engineered substrates that replace lost tissue rather than just facilitate wound healing. Autograft is currently the preferred option, but in many instances, there is an insufficient amount of tissue available for grafting, or the patient's condition precludes the use of autograft. Allografts and xenografts can provide a temporary coverage option, but they come with issues regarding rejection, and possible disease transfer, availability, as well as cultural and ethical considerations [26]. A gold standard in chronic wound management is the use of a full split-thickness autograft from a donor site and grafting it over the compromised region. Other options for chronic wounds are the use of donor keratinocytes or cultured epithelial autografts. This has been applied in the form of confluent sheets of cells applied directly to the wound bed or onto a pre-prepared wound base made of allograft dermis. Limitations of tissue grafting are the quantity of donor skin available, the risk of complications including pain and infection, high cost, and the need for multiple applications [5]. To avoid some of these problems bioengineered skin substitutes both biosynthetic and cultured autologous engineered skin is available to provide temporary or permanent coverage in large quantities and negligible risk of infection or immunologic issues. The main limitation of these products is their costs and still limited efficacy.

Formulation of LL37-AuNPs:

Previously, in our group, we had prepared LL37-AuNPs at room temperature in a one-step method [29]. LL37-AuNPs have an Au core and a hydrophilic cationic LL37 peptide shell. We have selected AuNPs because it is relatively easy the immobilization of high concentrations of LL37 per surface area, and the modification of their properties (including size, charge, and morphology) [30]. The production of our NP formulation involved only a processing step (which included the mixing of the peptide with gold salts, at room temperature) for one day followed by a centrifugation step. The simplicity of the process makes possible the large-scale production of LL37-conjugated NPs at a relatively low cost. We hypothesize that LL37-conjugated NPs have enhanced wound-healing properties than LL37 peptide because they prolong the biological activity of the peptide.

Design of LL37-AuNP coated dressing:

Although several research works have been done to evaluate the biological and biomedical application of biomolecule coated AuNPs, the biological fate of AuNPs is not known once they are administered in animals or the human body. Importantly, AuNPs are not approved by the FDA as a drug or a drug carrier. Our hypothesis is that LL37-AuNPs can be strongly coated on a commercially available wound dressing (polyurethane) without compromising the activity of LL37-AuNPs or leaching of LL37-AuNPs from the dressing.

MATERIALS AND METHODS

MATERIALS AND METHODS

Cell Culture

Human keratinocyte Cell Line (HaCat cell line, CLS, Heidelberg, Germany) was cultured in Dulbecco's Modification of Eagles's Medium (DMEM), with 4.5 g/L glucose & L-Glutamine without sodium pyruvate (500 mL, CORNING, USA), supplemented with 10% (V|V) fetal bovine serum (FBS, Gibco, ThermoFisher Scientific, USA) and 1% (V|V) penicillin and streptomycin, until reaching approximately 80%-90% confluence. The cell culture was maintained in an incubator having conditions of 37°C and 5% CO_2.

Cell Viability Assay

HaCaT cells of a density of 2×10^4 cells per well in a 24 well plate were placed on the top of LL37-AuNP-PU dressing, polymer adhesive coated PU dressing and PU dressing and cultured for 4 and 24h. Similarly, HaCat cells were also incubated with soluble LL37 peptide (20 µg/mL). HaCat cells grown on tissue culture polystyrene plate (TCPS) were considered as control. After 4 and 24h, HaCat cells were trypsinized and cells were collected for CellTiter-Glo® luminescent cell viability assay (Promega) to assess the ATP production in cells according to the supplier's instructions. In general, 100 µL of trypsinized cells were mixed with 100 µL of the CellTiter-Glo® reagent and the reaction was left for 10 minutes at room temperature in order to stabilize the luminescent signal as described in the protocol. After the incubation period, the luminescent signal was recorded using a Microplate Reader Synergy H1 (BioTek) Spectrophotometer. A Standard Curve using different concentrations of HaCat cells was made to evaluate the cell viability.

Animal Study

6-7 week old db/db mice (Charles River) were anesthetized with isoflurane and the hair in the dorsal area was shaved using an electric shaver and depilatory cream before the surgery. 6 mm^2 round-shaped full-thickness excision wounds were made using the skin biopsy punch on both sides of the dorsal side of each mouse. 12 mice were assigned per groups and LL37-AuNP-PU and PU dressings were placed randomly on either side of the wounds. The dressings were fixed on the mice using transparent Tegaderm adhesive dressing. At days 6 and 14, mice were sacrificed and wound tissues were collected for immunofluorescence and qRT-PCR analyses.

Immunofluorescence analysis of wound tissue samples

Wound tissues, obtained from wounds treated with LL37-AuNP-PU dressing (n=6) and PU dressings (n=6) for days 6 and 14, were fixed in paraffin. Before immunofluorescence analysis, wound samples went through deparaffinization, in which the wound samples were submersed in xylene solution for 10 minutes followed by submersion in xylene solution for 10 minutes. Then, the samples were submerged serially in 100% ethanol solution (5 minutes followed by 5 minutes), 96% ethanol solution (5 minutes). 70% ethanol (5 minutes) and finally in MilliQ water (5 minutes). The antigen retrieval of samples was performed using 10 mM sodium citrate buffer (pH 6) containing 0,05% Tween 20. Next, the samples were washed 3 times in PBS (1X) and permeabilized in 0,2% Triton-X 100 for 10 minutes at room temperature. Following the permeabilization, the samples were once again washed in PBS (1X) and incubated for 1 hour at room temperature with the blocking agent BSA 5% (w|v). Promptly after the blocking stage, the tissue samples were again incubated, overnight at 4°C, in the following primary antibodies: **MMR/CD206** (Rat, 1:8; *R&D Systems*, EUA), **CD80** (rabbit, 1:100; *Abcam*, UK), **Keratin 14** (rabbit, 1:1000; *BioLegend*, USA), **Keratin 5** (chicken, 1:200; *BioLegend*, USA). After the incubation with the primary antibodies, the tissue samples were washed in PBS (1X) and incubated in the following antibodies: **Alexa Fluor 488 Donkey Anti-Rat** (1:800; *ThermoFisher Scientific*, USA), **Cy^{TM}3 – Conjugated AffiniPure Goat Anti-Rabbit** (1:800, *Jackson Immuno Research*, UK), **Alexa 633 Goat Anti-Rabbit** (1:500; *Thermo Fisher Scientific,* USA) and **Alexa 488 Goat Anti-Chicken** (1:500; *Thermo Fisher Scientific*, USA) for 1 hour at room temperature in a dark room. The samples were washed again in PBS (1X) and incubated in 4',6'-diamino-2-fenil-indol (2 µg/mL, DAPI, Sigma) for 10 minutes and re-washed in PBS. The images were acquired using the INCell Analyzer 2000 (ThermoFisher Scientific, USA), in the Cy3, Cy5, FITC and DAPI channels, and then analyzed using the Image-J Software (National Institutes of Health, USA).

For the quantification of K14 and K5 expressing cells, the ratio of the targeted K14 or K5 expressing cells with the total number of the cells was estimated. We quantified the K14 and K5 expressing cells on the wound gap and proliferative areas of the wound (wound edges). In addition, the thickness of the proliferative area of the wound and wound gap was measured. We took 15 measurements and calculated the average of the measurements. We also calculated the fluorescence intensity of the presence of the K14 or K5 expressing cells using the following formula: $Ir = Ar\ (I_{Ar} - I_{background})^*$

*(Ar= Area of intensity; I_{ar}= mean of interest; $I_{background}$= intensity of the background).

For the quantification of the pro-inflammatory macrophages (M1 macrophages) and anti-inflammatory macrophages (M2 macrophages), we did a ratio of all the $CD80^+$ or $CD206^+$ positive-labeled cells and the total number of cells in wound tissue samples.

In addition, we calculated the percentage of co-localization between M1 macrophages and M2 macrophages, using the JaCoP plugin (Just another Colocalization Plugin) on ImageJ software, in order to observe the transition of M1 subtype to M2 subtype of macrophages in days 6 wound tissues.

Real-Time Quantitative Reverse transcription PCR (qRT-PCR)

The gene expression analysis was performed to estimate the presence of different cytokines in wounds treated with LL37-AuNP-PU and PU dressings. The targeted genes were: Interleukin-1 (IL-1), TNF-α, interleukin-6 (IL-6), interferon Gamma (IFN-γ) and TGF-β.

Total RNA extraction of frozen wound tissues was performed using the RNeasy® Fibrous Tissue Mini kit (QIAGEN, Germany), accordingly to the manufacturer recommendation, and the quantification of total RNA, as well as, the purification levels were performed using the NanoDrop 2000 Spectrophotometer (ThermoFisher Scientific). Following the RNA extraction, the cDNA synthesis was performed using the qScript® cDNA Supermix kit (Quantabio, USA), also according to the manufacturer's recommendation. The samples were amplified for 5 minutes at 25°C, 30 minutes at 42°C, 5 minutes at 85°C and held at 4°C in the CFX Connect Real-Time PCR Detection System (BioRad, USA).

Lastly, the qRT-PCR protocol used was the NZYSpeedy qPCR Green Master Mix (2x), ROX (nzytech, Portugal). The qRT-PCR analysis was run for 40 cycles in the CFX Connect Real-Time PCR Detection System (BioRad, USA). The minimal cycle threshold (Ct) values were automatically calculated using the BioRad CFX Maestro software and the quantification of the targeted genes was normalized to the GAPDH gene using the Livak Method (Fold difference in the expression= $2^{-\Delta\Delta Ct}$).

Table 1: Primers of target genes and reference gene used for qRT-PCR analysis are listed below:

	Forward Sequence	Reverse Sequence
GADPH	AGCCACATCGCTCAGACACC	GTACTCAGCGCCAGCATCG
IL-1β	5'- GCTCCGAGATGAACAACA	5' - GAGAATATCACTTGTTGGTGA
TNF-α	5´- GTCTCAGCCTCTTCTATT	5'- CCATTTGGGAACTTCTCATC
IL-6	5' - ACCTGTCTATACCACTTCAC	5' - GGCAAATTTCCTGATTATATCCA
IFN-γ	5' - TGAGTATTGCCAAGTTTGAG	5' – CTTATTGGGACAATTCTCTTCC
TGF-β	5' - GGATACCAACTATTGCTTCAG	5' -TGTCCAGGCTCCAAATATAG

Statistical Analysis

One-way ANOVA statistical analyses were performed for paired comparisons of means using the GraphPad Prism 6.0 Software. Results were considered significant when $P<0.05$. Data are shown as mean ± SEM.

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

Biocompatibility of LL37-AuNP-PU dressing against HaCaT Cells.

To evaluate the cytotoxicity of dressings, HaCaT cells were cultured on the top of LL37-AuNP-PU, polymer adhesive-PU and PU dressings for 4 and 24 h and then cell viability were performed using ATP assay (**Figure 2**). As controls, we used HaCaT cultured on TCPS and in the presence of soluble LL37 peptides. There was no statically significant difference in HaCaT cells viability after incubation with different dressings for 4 and 24 h, indicating that LL37-AuNP-PU dressing is biocompatible to HaCaT cells involved in wound healing. However, a little reduction in cell viability was observed with polymer adhesive-PU dressing after 24 h of incubation. Interestingly, 20 μg/mL of soluble LL37 equivalent to the conjugated LL37 peptides on LL37-AuNP-PU dressing does not exert cytotoxic effect to HaCaT cells (**Figure 2)**.

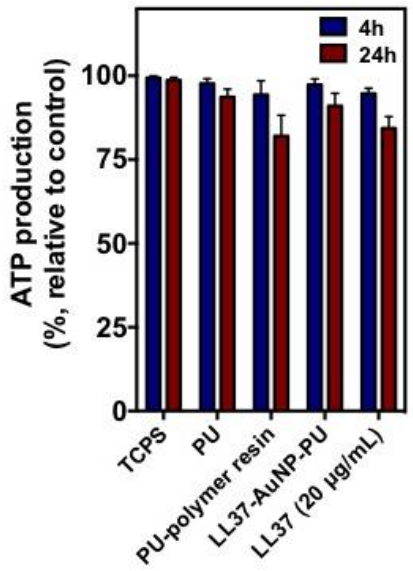


Figure 2: Cell viability study of HaCaT cells incubated with PU, polymer adhesive-PU and LL37-AuNP-PU dressings along with soluble LL37 peptide. TCPS represents HaCaT cells grown on tissue culture polystyrene 24 well plate.

Wound Healing Properties of LL37-AuNP-PU dressing

Previously in our lab, the wound healing potential of LL37-AuNPs coated PU dressings and bare PU dressings were evaluated in diabetic (db/db) mice. In general, wounds made on

the dorsal side of mice were treated with LL37-AuNP -PU and PU dressings for 14 days. The wound closure was visually monitored by acquiring the photos of the wounded beds and the rate of wound closure was quantified by measuring the wound bed area at an interval of 3 days for the total 14 days. Wound samples were collected at days 6 and 14 and preserved to perform immunofluorescence and qRT-PCR analysis to understand the mechanism of wound healing. The goal of this project is to understand the mechanism of wound healing by LL37-AuNP-PU dressing. In this project, I have performed immunofluorescent analysis of wound samples to evaluate the expression of keratin 14 and 5 (K14/5) in day 6 wound samples. I have also studied the presence of different subtypes of macrophages in wound samples of days 6 and 14 along with rt-PCR analysis to evaluate the expression of different cytokines, which help the rapid closure of the wound.

Analysis of expression of Keratin 14 and 5 (K14/5)

We performed immunofluorescence analysis of day 6 wound tissues to understand the expression of K14/5. K14/5 are highly expressed in the basal layer of the epidermis and required for normal development and functioning of basal cells. Their expression is down-regulated and gradually reduced as these cells moved upward and differentiate in wounds. Importantly, the study of K14 and K5 is fundamental for understanding the wound healing mechanism, since they form a proper sheet of basal keratinocytes to facilitate the migration of other cells and keratinocyte subtypes into the wound bed [16, 31]. The result shows the high expression of K14/5 in wound treated with LL37-Au NP dressings compared to PU dressing (**Figures 3 and 4**). Moreover, the expressions of K14 and K5 are more in proliferative edges and there is thicker keratin deposition at the edges of wounds (**Figures 1 and 2**). Based on K14 expression, LL37-AuNP-PU dressings promote the increase of proliferative length, reduction of wound gap and the more keratin deposition in the proliferative side of wounds as compared to PU dressings (**Figure 2**).

Overall this result shows that LL37-AuNP-PU dressings promote wound closure by promoting the migration of keratinocyte cells in wounds as compared to PU dressings.

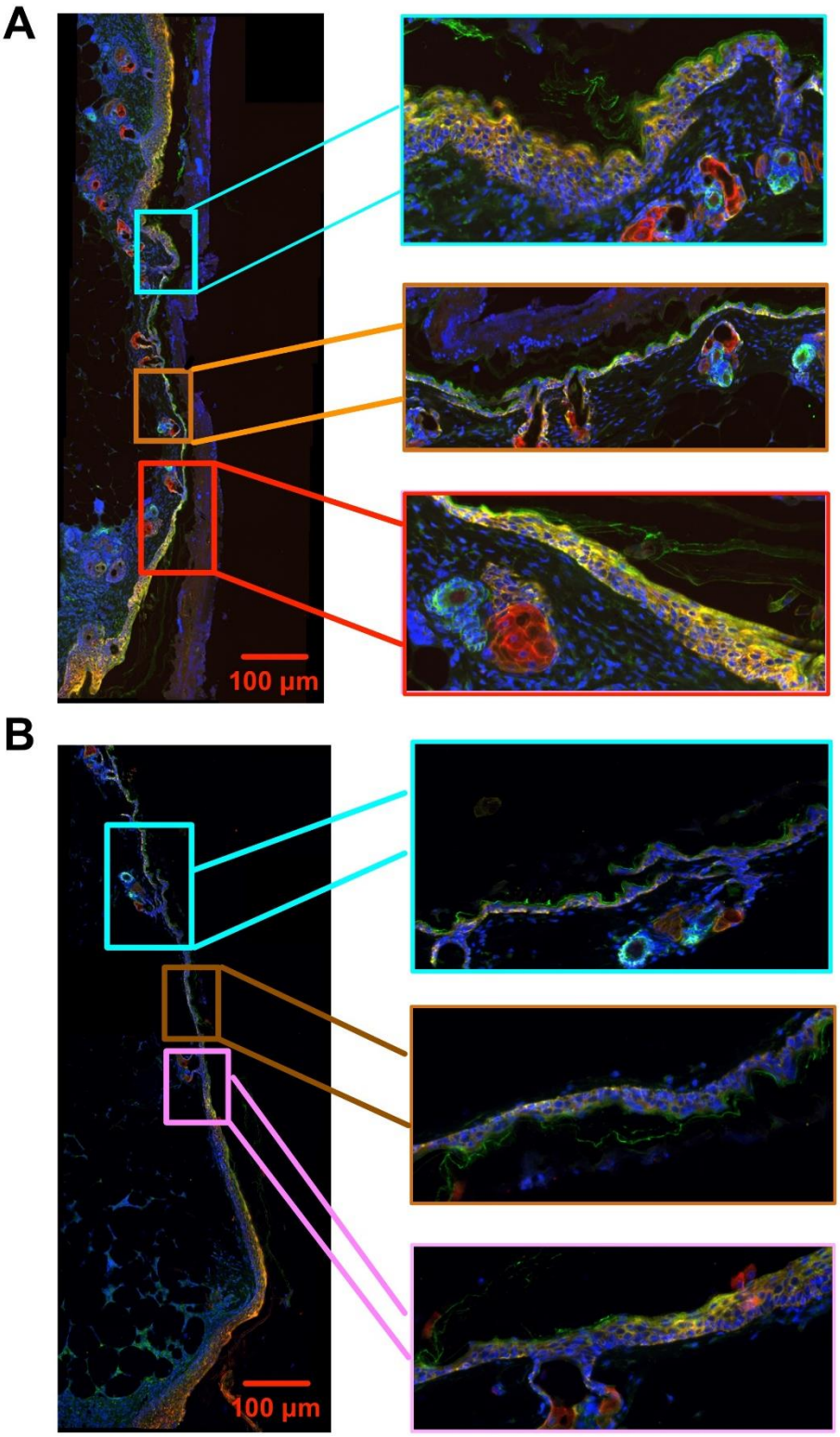


Figure 3: Immunofluorescence analyses of day 6 wound tissues to show the expression of K14 and 5 after treating with LL37-AuNP-PU (A) and PU (B) dressings. Red and green stained cells represent the expression of keratin 14 and 5 respectively.

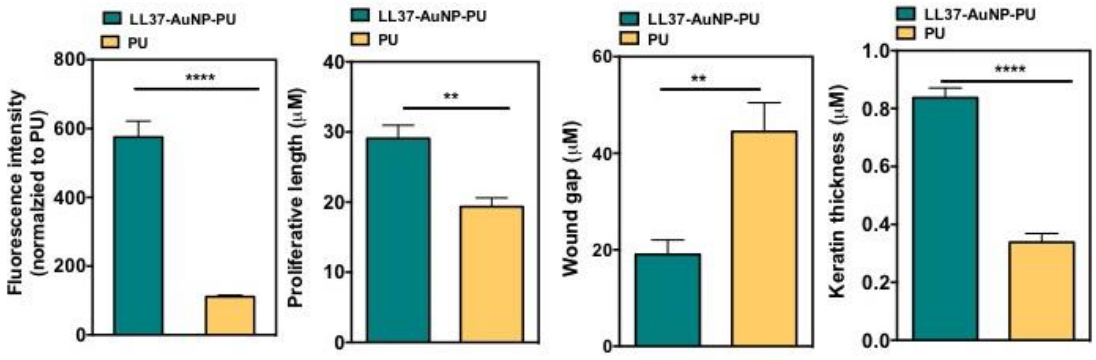


Figure 4: Quantification of fluorescence intensity, proliferative length, wound gap and thickness of keratin-based on the expression of K14. Results are average ± SEM, (n=6). Statistical analyses were performed by unpaired t-test. **P < 0.01 and ****P < 0.001.

Attenuation of the inflammatory response through regulating macrophages activation

It is well known that macrophages are the main inflammatory cells that are essential for effective wound healing and skin regeneration. Macrophages are considered the *"Orchestra Leader"* of the wound healing process since they are responsible for the resolution of the inflammatory phase, decontaminating the wound bed from infiltrating microorganisms and clearing apoptotic cells along with promoting the proliferation and tissue regeneration. During the progression of the normal wound healing process, there is a transition of macrophage phenotype from pro-inflammatory (M1) in early-stage to anti-inflammatory (M2) in late-stage to coordinate the regeneration of the skin. Importantly, macrophages hyperpolarize towards both M1 and M2 in early wound healing in the normal conditions, opposite to the continuous expression of the M1 phenotype in the late stage of healing in diabetic wound conditions. Moreover, the activation of inflammatory cells is irregular in diabetic conditions. In order to study the level of macrophages present in the wound tissue, we marked the M1 and M2 subtypes with a CD80 and MMR/CD206 antibodies respectively. To quantify the number of M1 macrophages present in the wound, the following equation was used: $\% M1\ Cells = \frac{Total\ number\ of\ M1\ Cells}{Total\ number\ of\ cells}$. The same procedure was used to quantify M2 cells.

Immunofluorescence analysis of day 6 wound tissues shows the expression of both M1 (single positive CD80) and M2 (single positive CD206) phenotypes of macrophages, however the expression of M1 is slightly higher than the M2 phenotype in LL37-AuNP-PU dressings than the PU dressings treated wounds (**Figure 5A and Supplementary Figures 1 and 2**). The presence of double-positive CD80/CD206 cells indicates the switching of macrophage

phenotypes from M1 to M2 in day 6 wound tissues (**Figures 5B-D**). Although the mixed population of M1 and M2 phenotypes are found in wounds tissue, LL37-AuNP-PU dressings stimulate a higher level of M1 to M2 phenotype switching as compared to PU dressings (**Figure 5B**).

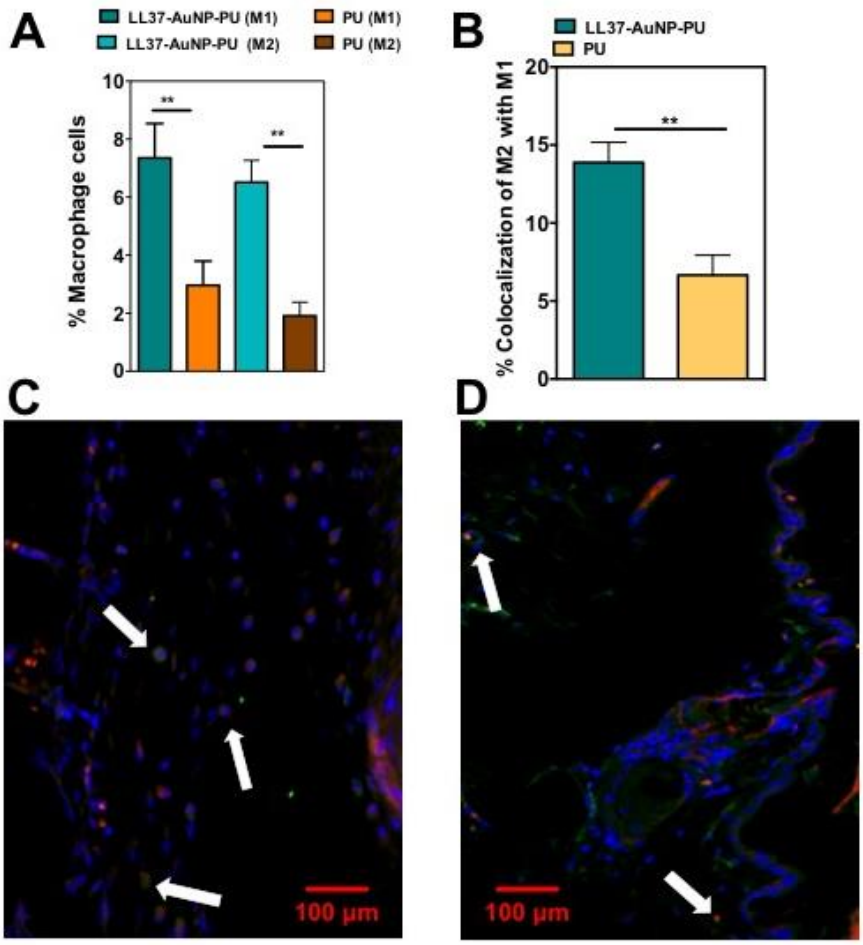


Figure 5: Quantification of M1 and M2 phenotype macrophages in day 6 wounds treated with LL37-AuNP-PU and PU dressings. Quantification (**B**) and immunofluorescence analysis of co-localization of M1 and M2 phenotype macrophages in day 6 wounds treated with LL37-AuNP-PU (**C**) and PU (**D**) dressings. Arrows show colocalized M1 and M2 cells. Results are average ± SEM, (n=6). Statistical analyses were performed by unpaired t-test, $^{**}P < 0.01$ and one way ANOVA test, $^{**}P < 0.05$.

Immunofluorescence analysis showed the presence of M1 and M2 macrophages in day 14 wound tissues (**Supplementary Figures 3 and 4**). Furthermore, we have quantified the levels of M1 and M2 macrophage phenotypes in day 14 wound tissues. Curiously, we observed that the number of M1 macrophages in day 14 wound tissues is higher than the day 6 wound tissues for PU dressing treated wounds, however, no such effect was found for LL37-AuNP-PU dressing treated wounds (**Figures 5A and 6**). Additionally, the level of M1 macrophages is less in wounds treated with LL37-AuNP-PU as compared to wounds treated with the PU dressings for day 14 wound tissues (**Figure 6**). Importantly, as expected we observed the increased level of M2 macrophages in day 14 wound tissues as compared to day 6 wound tissues, although the level of M2 macrophages is approximately the same in both conditions (LL37-AuNP-PU and PU dressings) for day 14 wound tissues (**Figures 5A and 6**). We believe that further immunofluorescence and colocalization analyses of M1 and M2 macrophages for days 14 wound samples are needed in order to confirm this result.

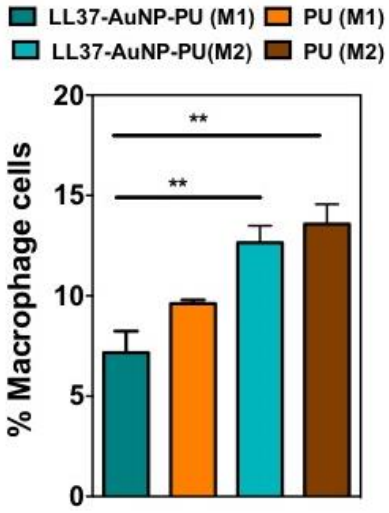


Figure 6: Quantification of M1 and M2 macrophages in day 14 wounds treated with LL37-AuNP-PU and PU dressings. Statistical analyses were performed by one way ANOVA test, **P < 0.01.

Overall, this result suggests that at day 14 post-injury, there is an anti-inflammatory environment in wounds treated with LL37-AuNP-PU dressings, which promotes the proliferation and remodeling stages, leading to the rapid healing of the wound.

qRT-PCR analysis of cytokines expressed in wounds

We performed the gene-level analysis of days 6 and 14 wound tissues in order to study the inflammatory status such as the pro-inflammatory and anti-inflammatory cytokines expressed in wounds. The presence of cytokines in wounds also indicates the presence of M1 and M2 phenotypes of macrophages. We performed a qRT-PCR analysis of the following genes: Interleukin-1β (IL-1β), Interleukin-6 (IL-6), Interferon-ϒ (IFN-ϒ), Tumor Necrosis Factor-alpha (TNF-α) and Transforming Growth Factor-beta (TGF-β), because they play a very important role in an effective wound healing process by regulating all the stages of wound healing. Before discussing the results obtained, we have explained the role of different cytokines in wound healing.

Role of Transforming Growth Factors-β (TGF-β) in Wound Healing:

TGF-β is known to be the predominant cytokine throughout the process of every stage of wound healing. TGF-β is generally produced by macrophage, fibroblast, and platelets. In the early stages of wound healing (Inflammation stage), TGF-β is responsible for promoting the migration of inflammatory cells to the wound bed, mainly neutrophils, as well as, promoting the differentiation of local monocytes into macrophages in order to increase the inflammatory response. Additionally, TGF-β up-regulates the vascular endothelial growth factor (VEGF), which is one of the growth factors responsible for the proliferation of endothelial cells and the initiation of angiogenesis. In addition, TGF-β is also responsible for the conversion of fibroblast into myofibroblast, facilitating the rapid wound closure. In a chronic wound condition, the level of TGF-β becomes very low, because they are degraded by proteolytic enzymes (eg: Neutrophil elastase), reducing significantly their bioavailability and therefore bioactivity.

Pro-inflammatory Cytokines role in Wound Healing:

IL-1β is produced by neutrophils, macrophages, keratinocytes, and monocytes. Upon injury, pre-stored IL-1β in keratinocytes is released and promotes the proliferation and migration of more keratinocytes into the wound bed. In addition, IL-1β also acts as a chemo-attractant for the migration of neutrophils and promotes the adhesion of neutrophils to the endothelial cells by activating adhesion molecules.

In the case of IL-6, they are produced by neutrophils and monocytes. Additionally, IL-6 is responsible for the recruitment and migration of more macrophages and fibroblasts into

the wound bed in the early stages of wound healing and is also responsible for the migration of neutrophils to the same area. IL-6 also promotes mitogenesis and proliferation of keratinocytes in the later stages of wound healing (such as in the re-epithelization stage).

Similarly to IL-1β, TNF-α promotes the migration of neutrophils to the wound bed since they act as neutrophils chemo-attractant and stimulates the adherence of neutrophils to the endothelial cells. Besides that, TNF-α is responsible for the recruitment of more macrophages and they induce MMP synthesis in keratinocytes, macrophages, and fibroblasts in the same manner as IL-1β. A low level of TNF-α controls inflammation and promotes reepithelization. On the contrary, a higher level of TNF-α suppresses the TIMP's expression, while increasing the production of MMP's, which results in a non-healing wound condition. In chronic wounds, the levels of TNF-α and IL-1β are elevated, promoting a more inflammatory environment, which in turn delays wound healing.

Finally, IFN-Ɣ is an endogenous cytokine produced predominantly by T-lymphocytes and NK cells. IFN-Ɣ up-regulates macrophage expression and is an inducer of iNOS release. However, several studies show that IFN-Ɣ can inhibit collagen synthesis by fibroblast in in-vitro condition, and exogenous administration of IFN-Ɣ promotes the decrease of collagen accumulation and disrupts wound strength in the in-vivo model. It is also showed that endogenous IFN-Ɣ negatively regulates the biological activity of TGF-β in wound healing, resulting in delayed wound closure [16, 34, 35, 36, 37, 38].

The qRT-PCR analysis showed that LL37-AuNP-PU dressings suppressed the production of IL-1β, IL6, TNF-α and IFN-Ɣ as compared to the PU dressings in day 6 wound tissues, indicating that there is no prolonged inflammation in wounds treated with the LL37-AuNP-PU dressing (**Figure 7**). Importantly, there is no significant increased in inflammatory cytokines in wounds treated with LL37-AuNP-PU dressings for 14 days. In a similar line, we did not observe an increased signal of inflammatory cytokines in wounds treated with the PU dressings from day 6 to day 14. On the other hand, there is an increase in the level of TGF-β wound treated with LL37-AuNP-PU dressings.

Overall, these results showed that the level of cytokine expression well-matched with the presence of M1 and M2 macrophages in different periods of wound healing. LL37-AuNP-PU dressing regulates the activation of macrophages and improves the progress of inflammatory stages to accelerate wound healing. In a chronic wound condition, it is typical to observe the increased levels of IL-1β, IL-6, and TNF-α and decreased levels of TGF-β. As

the results suggest, there is a less inflammatory environment in wound treated with LL37-AuNP-PU dressing, promoting the proliferative and remodeling stages of wound healing.

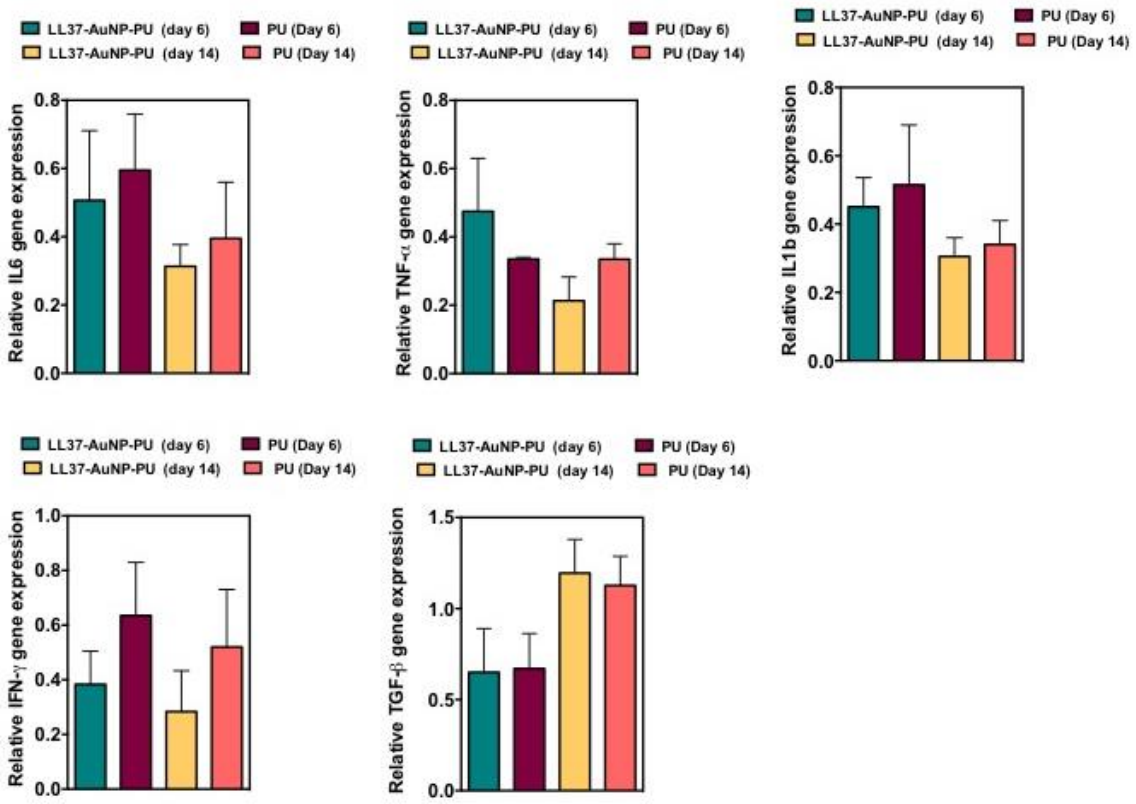


Figure 7: qRT-PCR analysis of IL6, TNF-α, IL1β, IFN- ϒ cytokines and TGF- β growth factor present in wounds treated with LL37-AuNP-PU. And PU dressings for days 6 and 14. Statistical analyses were performed using one way ANOVA test, however, no statistical significance was found.

CONCLUSIONS AND FUTURE PERSPECTIVES

CONCLUSIONS AND FUTURE PERSPECTIVES

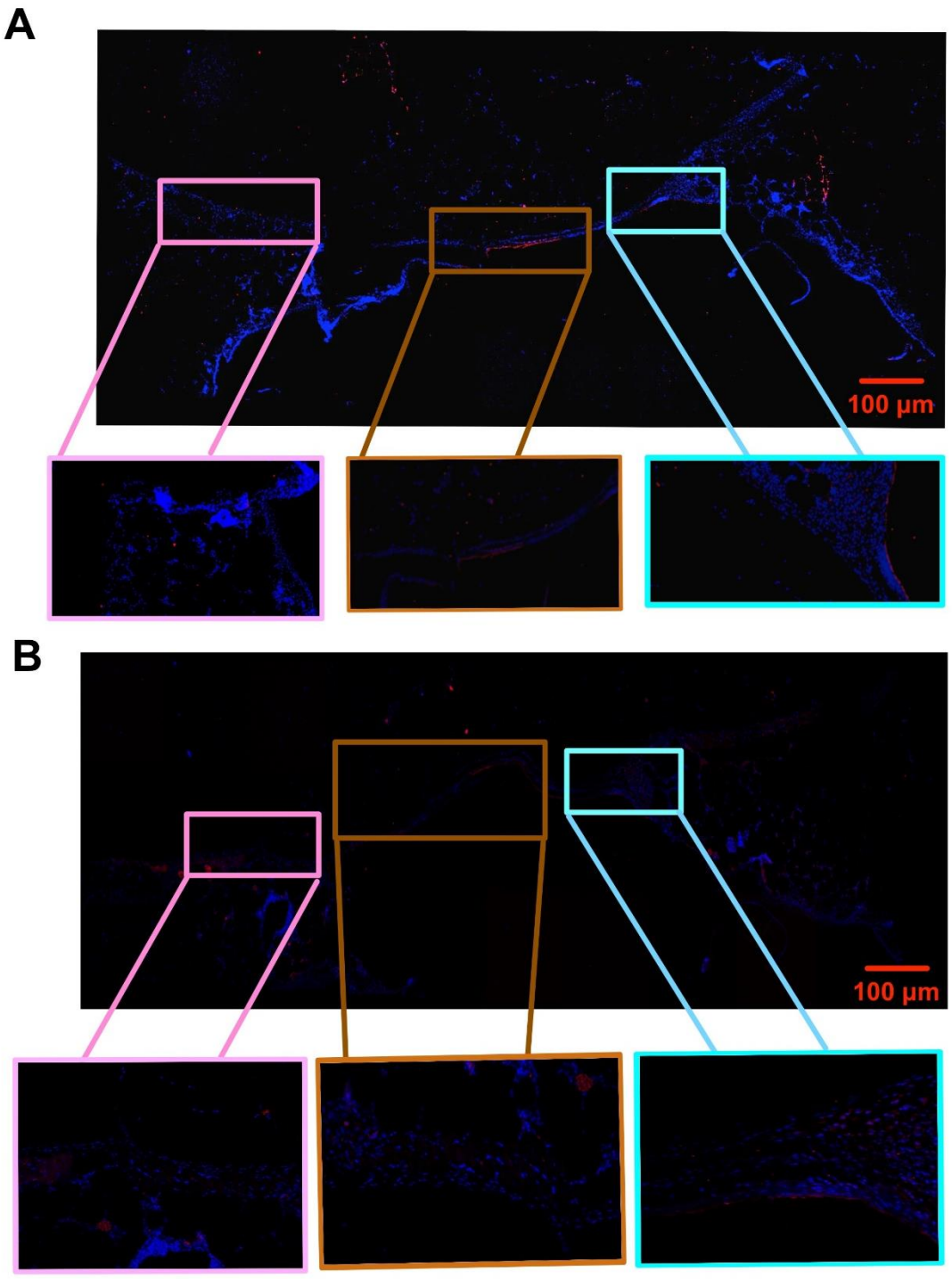
Understanding the pathophysiology of wound healing mechanism of various etiologies has been the target of many scientific studies in the past few years. Despite the increasing awareness and scientific studies explored in this subject, impaired wound healing and its medical complications remain one of the most prevalent and economically burdensome healthcare issues in the world. Current therapies are not the most effective but at the same time are expansive, indicating the need to develop new therapies for this unmet medical condition.

In my thesis, we studied the therapeutic effects of LL37-AuNP-PU dressing, which is a novel dressing developed in our lab. The advantage of this dressing is that LL37 present on the PU dressing promotes rapid wound healing along with the prevention of microbial infection. The main goal of this thesis was to studies the regenerative properties of LL37-AuNP-PU dressing on diabetic wounds and to evaluate the cytotoxicity of this dressing on HaCaT cells in *in-vitro* condition.

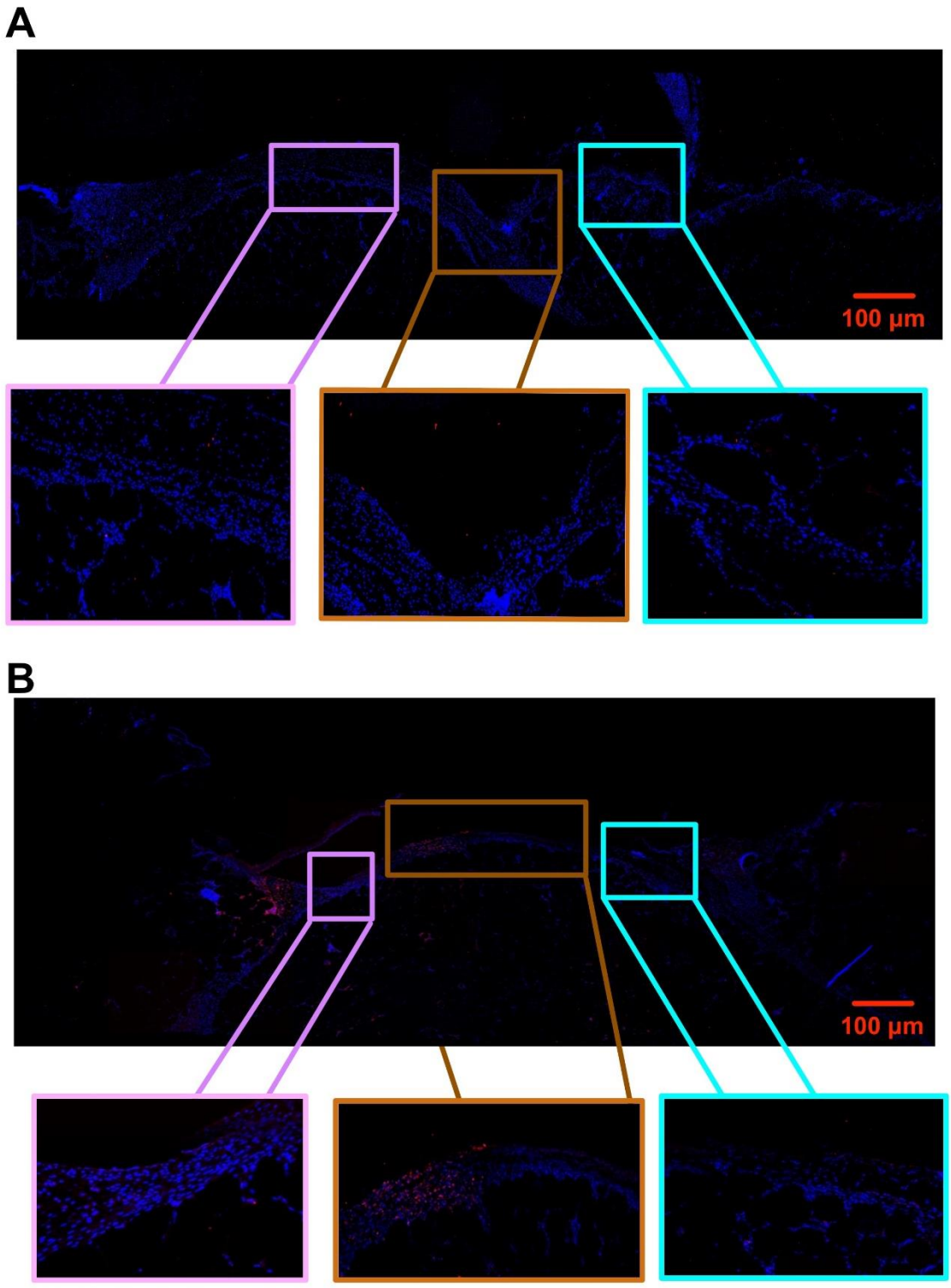
This work showed that there was no statically significant difference in HaCaT cells viability after incubating with LL37-AuNP-PU dressing for 4 and 24h, indicating that the LL37-AuNP-PU dressing is biocompatible to HaCaT cells involved in the wound healing. We also showed that soluble LL37 at a concentration of 20 µg/mL (equivalent to LL37 peptide present on the dressing) had no cytotoxic effect on HaCaT cells. Additionally, LL37-AuNP-PU dressings induced the expression of K14/5 in the proliferative edge and the gap of wounds, promoting the rapid wound closure.

Additionally, this study demonstrates that LL37-AuNP-PU dressing attracts macrophages in diabetic wounds and aids them to switch from M1 to M2 subtypes on day 6. Importantly, LL37-AuNP-PU dressing promotes high levels of M2 macrophage in day 6 and 14 wounds. Furthermore, qRT-PCR analysis well-aligned with the presence of M1 and M2 macrophages, in which pro-inflammatory cytokines decrease in wounds treated with LL37-AuNP-PU and PU dressings with healing time. Overall, LL-37AuNP-PU dressing regulates the activation of macrophages and improves the progression of the inflammatory stages of wound healing, promoting the proliferative and remodeling stages of wound healing. Finally, the prepared LL-37AuNP-PU dressings could be useful in treating chronic and diabetic wounds along with the prevention of bacterial infection.

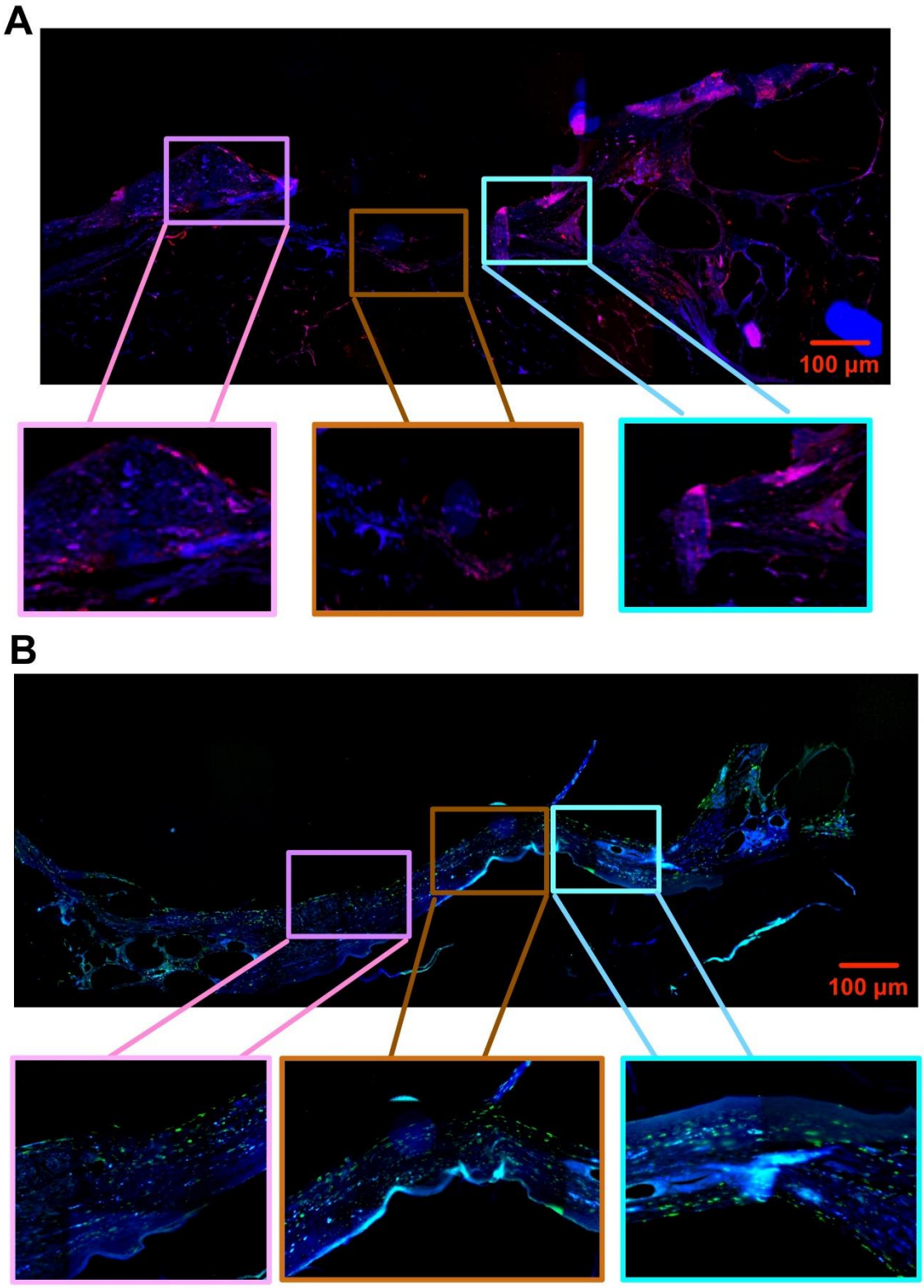
SUPPLEMENTARY FIGURES



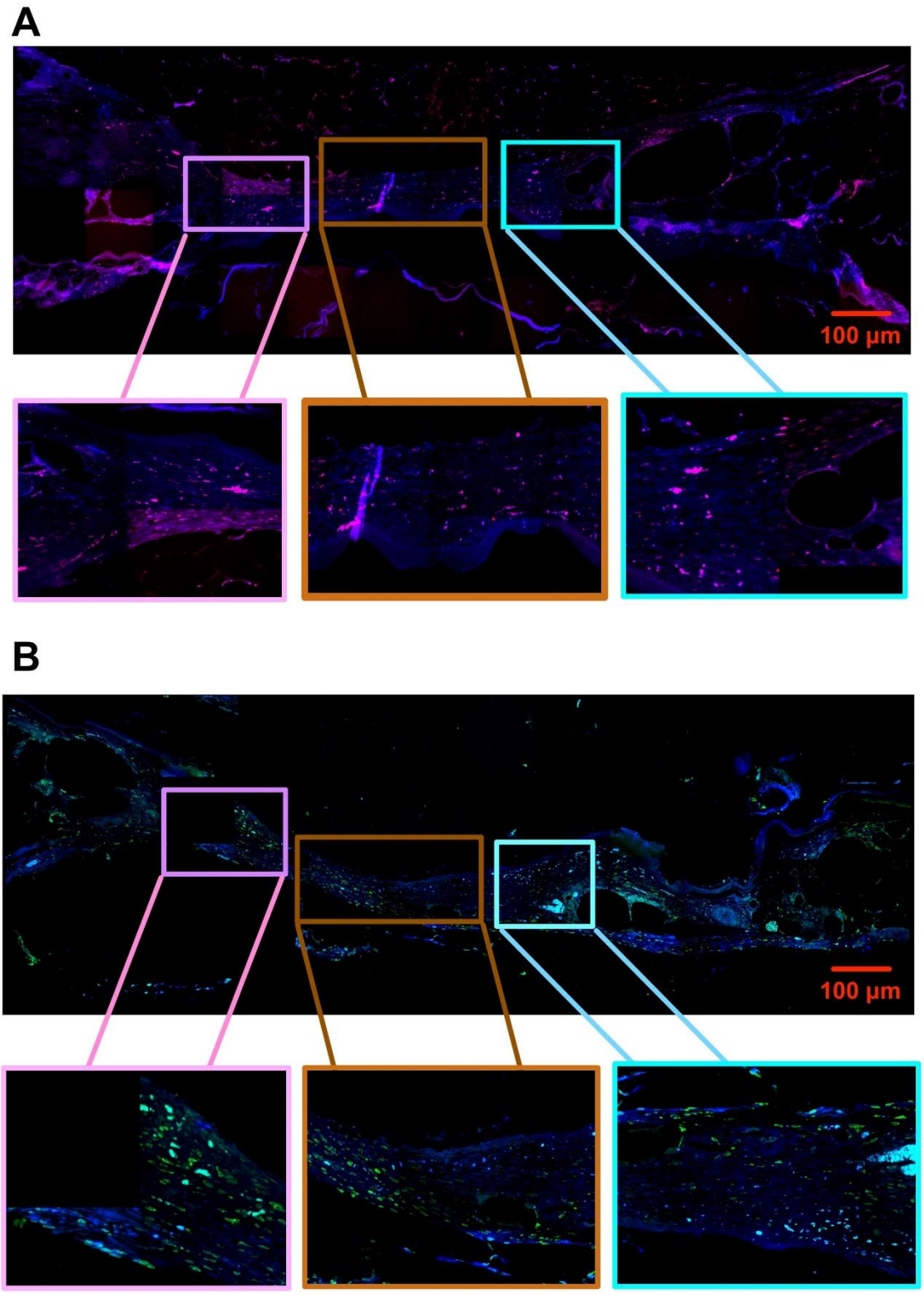
Supplementary Figure 1: Immunofluorescence analysis of M1 (**A**) M2 (**B**) phenotype macrophages in wounds treated with LL37-AuNP-PU dressings for 6 days. Blue stained cells correspond to the nucleus of cells; Red and green stained cells represent M1 and M2 macrophages respectively.



Supplementary Figure 2: Immunofluorescence analysis of M1 (**A**) M2 (**B**) phenotype macrophages in wounds treated with PU dressings for 6 days.



Supplementary Figure 3: Immunofluorescence analysis of M1 (**A**) M2 (**B**) phenotype macrophages in wounds treated with LL37-AuNP-PU dressings for 14 days. Blue stained cells correspond to the nucleus of cells. Red and green stained cells represent M1 and M2 macrophages respectively.



Supplementary Figure 4: Immunofluorescence analysis of M1 (**A**) M2 (**B**) phenotype macrophages in wounds treated with PU dressings for 14 days. Blue stained cells correspond to the nucleus of cells. The red and green stained cells represent M1 and M2 macrophages respectively.

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