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Groningen, The Netherlands

Cover: Xuan Li and Maria Isabel Patiño Vargas

Printed by: Ipskamp Printing

ISBN: 978-94-6421-555-7

university of groningen

UNIVERSIDAD DE ANTIOQUIA
1803

Engineering Human Skin Equivalents to Prevent Infections in Skin Wounds

PhD thesis

to obtain the degree of PhD of the
University of Groningen
on the authority of the
Rector Magnificus Prof. C. Wijmenga
and in accordance with
the decision by the College of Deans

and

to obtain the degree of PhD of the
University of Antioquia
on the authority of the
Rector Prof. J. Arboleda
and in accordance with
the decision by Corporation of Basic Biomedical Sciences Academic Council

Double PhD degree

This thesis will be defended in public on
Wednesday 17 November 2021 at 12.45 hours

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To my dear mom

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Chapter 1

General Introduction and Aim of this Thesis

Non-healing skin wounds are a significant socioeconomic burden due to their high prevalence worldwide. The inefficient recovery of autologous tissue in these wounds increases the susceptibility of patients to infections caused by microorganisms resistant to conventional antimicrobial therapies. These infections increase local tissue damage and delay wound healing, which leads to the development of septic processes that can be life-threatening. Therefore, it is necessary to develop novel approaches that prevent skin wound infections and, in turn, dispense with the use of antibiotics to reduce bacterial resistance.

SKIN FUNCTION AND STRUCTURE

The skin is the first defensive barrier of the human body against external pathogens and environmental insults.[1] It also maintains homeostasis by controlling the passage of water and electrolytes and regulates body temperature. Skin plays a role in the body's adaptation during movement since it is flexible enough to resist tearing and piercing.[2,3] Structurally, the skin consists of three main regions (from the outside in): the epidermis, dermis, and hypodermis (Figure 1).[4]

The epidermis

The epidermis is 0.1-0.2 mm thick, it does not contain blood vessels but receives nutrients through diffusion from the blood capillaries in the upper dermis. It is composed mainly of keratinocytes (90–95%), although other cells that play a functional role in the epidermis such as Langerhans cells, melanocytes, Merkel cells, and T lymphocytes are also present.[5]

Keratinocytes

As shown in Figure 1, the human epidermis is divided into five main layers: the stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum, and stratum basale. In these layers, keratinocytes are in a continuous state of proliferation, differentiation, and exfoliation from the deeper layers to the superficial.

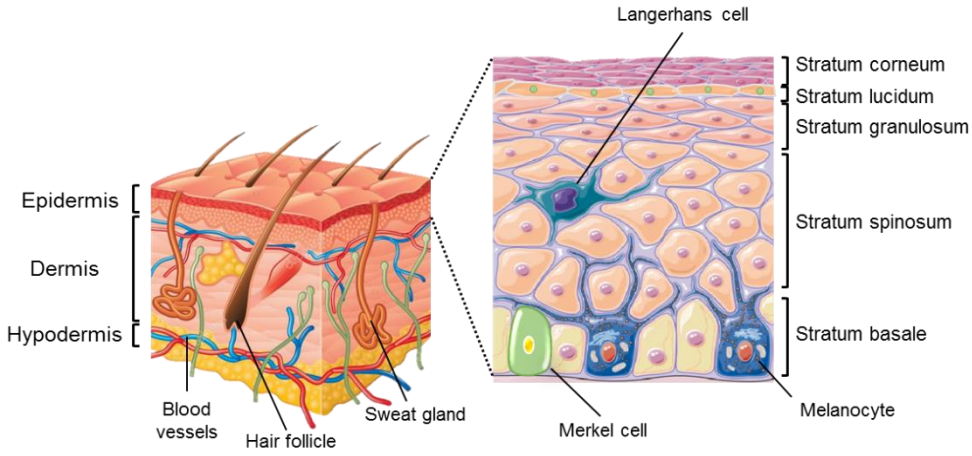

Figure 1. Schematic overview of the human skin and the epidermis layers. The epidermis is composed mainly of keratinocytes, but also contains non-epithelial cells, including Langerhans cells as well as melanocytes and Merkel cells. The dermis contains the skin appendages (sweat glands, sebaceous glands, and hair follicles), blood vessels, and nerve endings. Adapted from "Advanced Hydrogels as Wound Dressings" by S. Tavakoli, and A. S. Klar, 2020, Biomolecules, Aug 11;10(8):1169. Copyright © 2020 by the authors.[6]

The stratum basale (also known as the germinal stratum) is the deepest epidermal layer that gives rise to the keratinocytes of the epidermis. In the stratum basale, the basal cells remain attached to an underlying matrix and a proliferative state. Some daughter cells detach from the basal layer, migrate outward, and differentiate, forming the stratum spinosum. In this layer, cells start to produce and accumulate keratin proteins. The stratum granulosum is composed of flattened and basophilic keratinocytes, which contain shrunken nuclei and large, deeply basophilic keratohyalin granules in their cytoplasm. These cells eventually lose their nuclei and other cellular organelles, forming the stratum corneum (also known as corneocytes), the skin's outermost layer.

The stratum lucidum represents a transition from the stratum granulosum and stratum corneum. It is usually seen only in areas of thick skin such as the hand's palm or the soles of the foot and is composed of dead and flat keratinocytes that lie close to each other.

Melanocytes

Melanocytes are highly differentiated cells responsible for the production of pigment of the skin and hair in all mammals. They are located in the basal layer of the epidermis, at which they produce pigment granules called melanosomes that contain melanin. Melanosomes are transferred along the dendrites of the melanocyte surrounding the keratinocytes to protect their nuclei against the harmful effects of ultraviolet (UV) light.[7] The process of melanin production and transfer of melanosomes occurs steadily as the epidermis renews but can be sped up in response to UV exposure.

Langerhans cells

Langerhans cells are antigen-presenting dendritic cells of the skin, which constitute the immune barrier of the epidermis and are involved in contact allergy. These cells represent 3-5% of all nucleated epidermis' cells and are arranged in a network that occupies the interstices between neighboring keratinocytes.[8]

Merkel cells

Merkel cells constitute a population of postmitotic cells distributed along the dermo-epidermal junction. Most of these cells are associated with nerve endings, which is why they play a fundamental role in transmitting signals through synaptic contacts with somatosensory neurons. The amount of Merkel cells can vary between 0.2% and 5% in the epidermis. However, they are mainly found in hairy skin, taste buds, foot pads, and some glands (eccrine glands and sweat glands).[9]

The dermis

The dermis is the thickest of the skin layers, with a thickness of 3 mm. It primarily consists of extracellular matrix (ECM), which comprises fibrillar collagens and associated proteins that protect the body against mechanical injury by conferring elasticity and plasticity to the skin.[10] In young skin, fibroblasts produce and adhere to the dermal ECM, allowing these cells to spread and exert a mechanical force on the surrounding ECM.[11,12] In addition to fibroblasts, the dermis contains endothelial cells, mast cells, macrophages, dendritic cells, T-lymphocytes, and neutrophils.

The presence of a vascular network in this layer plays an essential role in wound repair by stimulating blood flow and providing oxygen and nutrients to the surrounding tissue. Furthermore, the blood vessels allow the circulation of immune cells to the wound site when the skin is compromised. Finally, the dermis supports the lymphatic system and nerve bundles that supply the sense of touch, heat, and pain.

The hypodermis

Below the dermis is the hypodermis or subcutaneous layer, constituting about 15-30% of the body mass. It is mainly composed of adipocytes that specialize in accumulating and storing fat. It functions as an insulating and cushion layer for the integumentary system, which is integrated by the skin and its accessory structures such as the nails, hair, and glands (sebaceous and sweat).[13]

SKIN WOUNDS

Wounds and their classification

Wounds are perturbations in the skin's structural integrity caused by mechanical trauma, surgical interventions, or burns. Skin healing can occur naturally, but in many cases, when the damage is extensive, the skin regeneration process may be affected, resulting in chronic wounds that eventually can lead to death. Burns are one of the most common injuries that affect large areas of skin. These can cause severe complications, including infections, which depending on the environment, size, and depth of the wound and the patient's immunocompetence, could delay wound healing and even be life-threatening. Therefore, immediate coverage of burns is needed to repair and regenerate the tissue. However, without the prevention of microbial colonization, wound treatment is practically impossible.[14]

Wounds can be classified according to their etiology, location, depth, tissue loss, or clinical appearance. The most common classification is performed according to the predominant tissue in the wound bed, which is divided into epidermal, partial-thickness (often divided into superficial partial-thickness and deep partial-thickness), and full-thickness wounds.[15] Epidermal wounds are

characterized by the loss of the epidermis only. Generally, this situation does not require treatment since the re-epithelialization process is fast and does not generate scarring. Partial-thickness wounds involve the epidermis and superficial dermis but can also involve more significant dermal damage. These types of wounds can be accompanied by epidermal blistering and severe pain. In this case, damaged skin heals in a self-limited manner by epithelialization from the wound's margin due to keratinocytes' migration and proliferation. Full-thickness wounds compromise the dermis, subcutaneous fat, and even bone. These wounds lead to cosmetic and functional defects and do not cure themselves as they cannot epithelialize correctly. Therefore, the treatment of most of these wounds requires skin grafts from healthy areas of the body.

Wound colonization and infection

From a microbiological point of view, the main function of intact skin is to prevent the colonization of the underlying tissue from microbial populations that live on the skin's surface.[16,17] Open skin wounds are colonized with bacteria, and optimal wound care is required to prevent progression to infection. Skin infections occur mainly in the outer layers. However, they can extend deeper into the underlying (subcutaneous) layer, increasing tissue damage and delaying recovery.[18] Normally, wound colonization is polymicrobial and involves numerous microorganisms that are potentially pathogenic. When the infection is established, the wound fails to heal, the patient suffers increased trauma, and the cost of treatment is higher.

Conditions of ischemia, hypoxia and necrosis become optimal for microbial growth in the affected tissue. Wound contaminating microorganisms come from three primary sources: the environment, the surrounding skin (which involves the normal skin microflora), and endogenous sources that mainly involve the gastrointestinal, oropharyngeal, and genitourinary mucous membranes.[19] Therefore, it could be speculated that the wounds with a sufficiently hypoxic environment, or in situations where the patient's immune response fails, are more susceptible to being colonized by several types of endogenous bacteria. However, aerobic or facultative pathogens such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and beta-hemolytic streptococci have been reported to be the main causes of infection and delayed healing both acute and chronic wounds.[19-23] Among these, *S. aureus* is considered the most

problematic bacterium in traumatic, surgical, and burn wound infections. Although the presence of such microorganisms frequently raises concern among healthcare professionals, their identification as the etiologic agent can only be confirmed if they are present as a pure monomicrobial flora.[20]

On the other hand, the progression of a wound to an infected state also implies the expression of virulence factors by one or more microorganisms, which activates the response of the host's innate immune system, causing a cascade of local and systemic responses. The severity of these infections depends on microbial and host factors. Microbial factors include the microbial load and the combined level of virulence expressed by the microorganisms involved. For example, it has been reported that healing in some types of wounds progressed only when the bacterial load was < 10^6 CFU/ml of wound fluid. Host factors include the type, size, and depth of the wound, as well as the general health conditions.[19,24]

TREATMENT OF WOUNDS

Wound Dressings

Different types of materials are currently available to be used as primary or secondary dressings to protect wounds from contamination. Traditional wound dressings include gauze, lint, bandages (natural or synthetic), and cotton. These dressings can favor the re-epithelialization process, promote angiogenesis by creating hypoxia to the compromised tissue, and prevent infections by lowering the pH of the wound bed.[25,26]

In clinical practice, dressings are indicated for minor wounds (e.g., superficial partial-thickness) that are generally clean, dry, and with mild exudate levels. Since traditional dressings do not provide a moist wound environment, they have been replaced by dressings with more advanced formulations, most of them based on synthetic polymers with high biocompatibility and biodegradability.[27]

Antimicrobial therapy

Historically, bacteriostatic or bactericidal antimicrobials have been administered both topically and systemically to treat skin wound infections.[28] The wide range of topical and/or systemic antibiotics include but are not limited to aminoglycosides, beta-lactams, lincosamides, macrolides, quinolones, tetracyclines, and cephalosporins.[29] These classes of antibiotics can obstruct some functions or metabolic pathways of bacteria either through inhibition of cell wall synthesis, interference with protein synthesis, and inhibition of genetic material synthesis.

Although many of these antibiotics are helpful in treating infected wounds, their repeated, and inappropriate usage has increased bacterial resistance, which represents one of the most challenging clinical challenges today.[30,31] About 70% of bacteria that cause skin wound infections have been reported to be resistant to at least one of the commonly used antibiotics.[32] In particular, *S. aureus* and *P. aeruginosa* strains were significantly resistant to antibiotic therapy, according to a study conducted on wound secretions with bacteriological identification.[17,28] Furthermore, the development of biofilms by infectious strains in chronic wounds, including diabetic ulcers, are starting to acquire resistance to almost all classes of antibiotics, leading to a need for increased efforts in the development of new antimicrobial therapies to prevent infections with minimal toxicity for the patient.[33]

Regarding topical antimicrobial treatments, the list of antibiotics is much smaller compared to systemic antibiotics; bacitracin, neomycin, polymyxin, mupirocin, and fusidic agents are some of the most frequently used.[34] These agents provide local antimicrobial activity, so they tend to have fewer systemic side effects.[35] However, their activity time is short, so most of them are used mainly to reduce the microbial burden on the skin surface in a prophylactic way. In addition, the success of topical therapies is challenged by harsh conditions in the wound microenvironment, especially when bacteria are embedded in an extracellular polysaccharide matrix, which protects them, thus impeding adequate concentrations of active antimicrobials at the site of infection.[36]

Alternative antimicrobial therapies

Among the strategies that have been developed to replace conventional antibiotics, the so-called nano-drug delivery systems have been frequently adopted to promote wound healing. In particular, for skin regeneration, hydrogels, nanofibers, nanoparticles, and their combinations are examples of these systems all under development.[37]

Antimicrobial-loaded hydrogels

Hydrogels are three-dimensional (3D) structures consisting of physically or chemically cross-linked bonds of hydrophilic polymers. These materials have been widely used in tissue engineering of the skin since their hydrophilic structures can favor the absorption of wound exudates and allow oxygen exchange to accelerate healing.[38,39] Hydrogels have highly hydrated polymeric networks and can bind several-fold more water compared to their dry weight, in addition, they can be molded into various shapes and sizes, which makes them a promising alternative for covering different types of wounds.

Hydrogels provide a 3D environment that can support cell growth to promote tissue regeneration through ECM production.[40] They offer a platform to load antibacterial agents (e.g., antibiotics, biological extracts, and synthetic antimicrobial drugs), growth factors, and/or biomacromolecules that promote skin healing. Nevertheless, despite their good performance in wound healing, the production of hydrogels continues to be a challenge due to high costs and harsh storage conditions. Some of the most common hydrogel components and their advantages and challenges are described in Table 1.

Table 1. Hydrogel materials for wound dressings: advantages and challenges.[40-43]

Hydrogel material		Advantages	Challenges
Natural polymers	Collagen	Favors cell attachment, good printing abilities.	No intrinsic antimicrobial activity, poor mechanical stability, slow gelation, easily clogs.
	Agarose	High mechanical strength, low cost, easy to manipulate.	No intrinsic antimicrobial activity, low cell adhesion.
	Chitosan	Antibacterial and antifungal properties.	Slow gelation, poor mechanical properties
	Silk fibroin	Biocompatible, degrades *in vivo*.	Costly to produce, cannot incorporate cells within.
	Alginate	Fast and stable gelling, low cost.	No intrinsic antimicrobial activity, poor cell attachment, easily clogs at high concentrations.
	Fibrin	Biocompatible, promotes angiogenesis, fast gelation.	No intrinsic antimicrobial activity, poor mechanical stability.
	Hyaluronic acid	Promotes proliferation and angiogenesis, fast gelation.	No intrinsic antimicrobial activity, degrades easily, poor mechanical stability.
Synthetic polymers	Polyethylene glycol	Tunable drug delivery profile, good when combined with other components.	Low cell proliferation, poor mechanical strength.
	Methacrylated gelatin/gelatin methacryloyl	Easily degradable, high mechanical strength.	Slow gelation, requires ultraviolet (UV) light which causes cell damage.

Nanofibers and nanoparticles

Over the past several years, nanofibers and nanoparticles have been produced as drug delivery systems for antimicrobial agents. Nanofibers can be woven into mats of different sizes, which cover and protect the injury.[44] These systems are manufactured mainly based on polymers, metals, and metallic oxides. One of the most cost-effective and efficient techniques for fabricating steady nanofibers is electrospinning, which makes it possible to produce nanofibers with diameters of the order of nanometers, ranging from 5 to 100 nm.[45] These nanofibers could have a high surface-to-volume ratio and porosity, which can increase the loading capacity and transport of the drug.

The use of nanoparticles has gained great interest for their ability to penetrate deep into the skin and disrupt bacterial growth.[46] Nanoparticle-based antimicrobial therapies can enhance the effects of antibiotics by manipulating the agent concentrations at the site of application. Silver nanoparticles (AgNP) and other compounds, such as nitric oxide and chitosan, have shown good antimicrobial properties.[47] In general, nanoparticles are a versatile treatment option with a wide variety of formulations that are relatively accessible in clinical practice. However, many of these materials have exhibited toxic effects in the body, limiting their use over long periods of time.

ANTIMICROBIAL PEPTIDES OF THE HUMAN SKIN – A STRONG ROLE FOR LL-37

Antimicrobial peptides (AMPs) are critical components of human innate immunity and contribute to the first line of defense against infection in the skin.[48] Most of them are cationic, amphipathic and have sizes between 12 and 100 amino acids.[49] The AMPs structure allows them to bind and disrupt the membranes of microorganisms.[50] In addition, AMPs can inhibit the microbial cell wall, nucleic acid, and protein biosynthesis. Despite AMPs being ancestral molecules, the development of generalized resistance by bacteria has been low, which is one of the main advantages of AMPs over existing antimicrobial agents.[51]

Defensins and cathelicidins, secreted primarily by keratinocytes, are two of the major classes of antimicrobial peptides in the skin. In humans, the only cathelicidin described to date is the LL-37 peptide. The human cathelicidin antimicrobial peptide (*CAMP*) gene encodes an inactive 18 kDa precursor protein (Hcap-18) that releases the active C-terminal 37 amino acid peptide LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES) during the post-translational process.[52-55] The peptide LL-37 has been shown to have antimicrobial and anti-biofilm activity against multiple Gram-positive and Gram-negative bacteria, making it a promising agent for treating skin wound infections. In order to understand the antimicrobial properties of LL-37, it is necessary to consider its features and secondary structure. The structure of LL-37 contains 54% hydrophilic residues with eleven basic and five acid groups, which gives it a net positive charge of +6 at physiological pH. LL-37 forms a random coil in aqueous solutions, but in the presence of membranes where the environment is lipophilic, it forms a cationic amphipathic α-helical structure.[52,56] This secondary structure is composed of 3 domains: an N-terminal α-helix followed by a C-terminal α-helix,

and a C-terminal tail (Figure 2). The LL-37 has a concave hydrophobic surface bordered by predominately positively charged residues, enabling the interaction with negatively charged molecules from the bacteria, such as lipopolysaccharides (LPS), DNA, or cell walls. The hydrophobic surface of LL-37 is determined by the presence of four aromatic phenylalanine (F) side chains that point in the same direction (Figure 2).

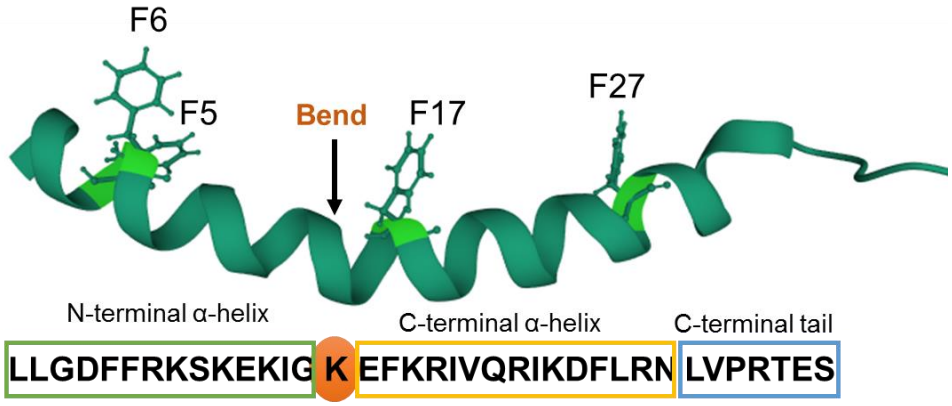

Figure 2. α -helical structure of the LL-37 peptide. The three-dimensional structure shows a helix-bend-helix conformation ending in a C-terminal tail. The image was created using RCSB Protein Data Bank.[57,58]

Beyond antimicrobial activity, it has been reported that the LL-37 peptide also plays an essential role in processes that promote tissue regeneration such as differentiation, proliferation,[59] and migration of keratinocytes and fibroblasts, it participates in angiogenesis[60] and wound closures.[61,62,63] In addition, the importance of these peptides in burn trauma has been demonstrated, where basal expression levels are not high enough to resist the infectious processes that these types of patients suffer.

SKIN GRAFTS

Autologous skin grafts have remained the standard of care for deep partial-thickness and full-thickness wounds. A graft is a section of skin of variable thickness and size, which is surgically removed from a donor site of healthy skin and applied to cover the damaged area. Concerning the thickness of the explant, a graft can be classified as split thickness and full thickness.[64,65] The first is further subdivided into thin (0.15-0.3 mm), intermediate (0.3-0.45 mm), and thick (0.45-0.6 mm) grafts. The full-thickness grafts contain the entire thickness of the epidermis and dermis (0.6 mm), and it only can be taken if the portion is small enough to be nourished by the surrounding capillaries.

One of the most troublesome morbidities associated with autologous skin grafts is the scarring at the donor site. Scar tissue in these areas is stiff, painful, dysfunctional, and can eventually contract, causing deformities. Therefore, numerous research efforts have focused on developing exogenous graft materials, such as cadaveric skin, xenografts, and artificial skin substitutes that could replace traditional autografts. However, at present, these options can only offer temporary wound coverage, and in many cases, autologous grafting remains necessary.

Based on the above, the availability of ready-to-use tissues, or the possibility to produce, in a short time, sufficient amounts of skin to treat these wounds, has become a challenge addressed in numerous investigations.

TISSUE-ENGINEERED HUMAN SKIN EQUIVALENTS (HSEs).

Tissue engineering is defined as an interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes to restore, maintain, or enhance tissue functions.[66] When this concept was introduced almost 20 years ago, the possibility of growing transplantable tissues in the laboratory, and generating HSEs became a promising alternative to treat acute and chronic skin wounds.[67,68]

Tissue engineering is based on three main components: cells, a scaffold, and a bioreactor.[69,70] First, the engineered tissue is matured *in vitro* by placing the cells with the scaffold in a bioreactor, which is a metabolically and mechanically supportive environment for cell proliferation and ECM production. Then, the

engineered tissue construct is implanted in the intended location of the body to start remodeling of the tissue *in vivo* and to recapitulate their normal structure or function. This *in vitro* and *in vivo* tissue formation and maturation happens due to multiple processes: 1) cell proliferation and differentiation; 2) ECM production and reorganization; 3) scaffold degradation; and 4) remodeling and formation of tissue.

In skin tissue engineering, the standard method for the production of HSEs involves seeding a natural or synthetic biodegradable matrix with cells, usually the cultivation of dermal fibroblasts within a hydrogel and a layer of keratinocytes seeded on top (Figure 3). The matrix provides a favorable environment for skin cell growth and ECM production.[71,72] Eventually, the matrix is degraded and replaced by cells and ECM, leading to the formation of the functional skin.

HSEs have been widely used in research as models for pharmacological testing to identify irritant, corrosive, and sensitization agents that come into contact with the human skin, without the need of using animal models.[73-75] Furthermore, HSEs can be used to investigate fundamental processes in the skin, such as the stimuli that lead to the formation of the epidermis, the molecular cross-talk between different cell types, the maintenance of stem cells, the process of wound healing, and the infection with different kinds of microorganisms.

One of the significant advantages of HSEs is that the cellular composition can be controllable by the researcher. Therefore, a certain cell population can be specifically integrated to determine their relevance in the biological process under investigation. On the other hand, in the field of clinical skin replacements, the main advantage of using HSEs is that they reduce or eliminate the need to take large areas of donor skin, which are commonly required for autologous grafts.[76,77] Additionally, they avoid recruiting volunteers for a specific study and eliminate the need for laboratory animals.[78-81]

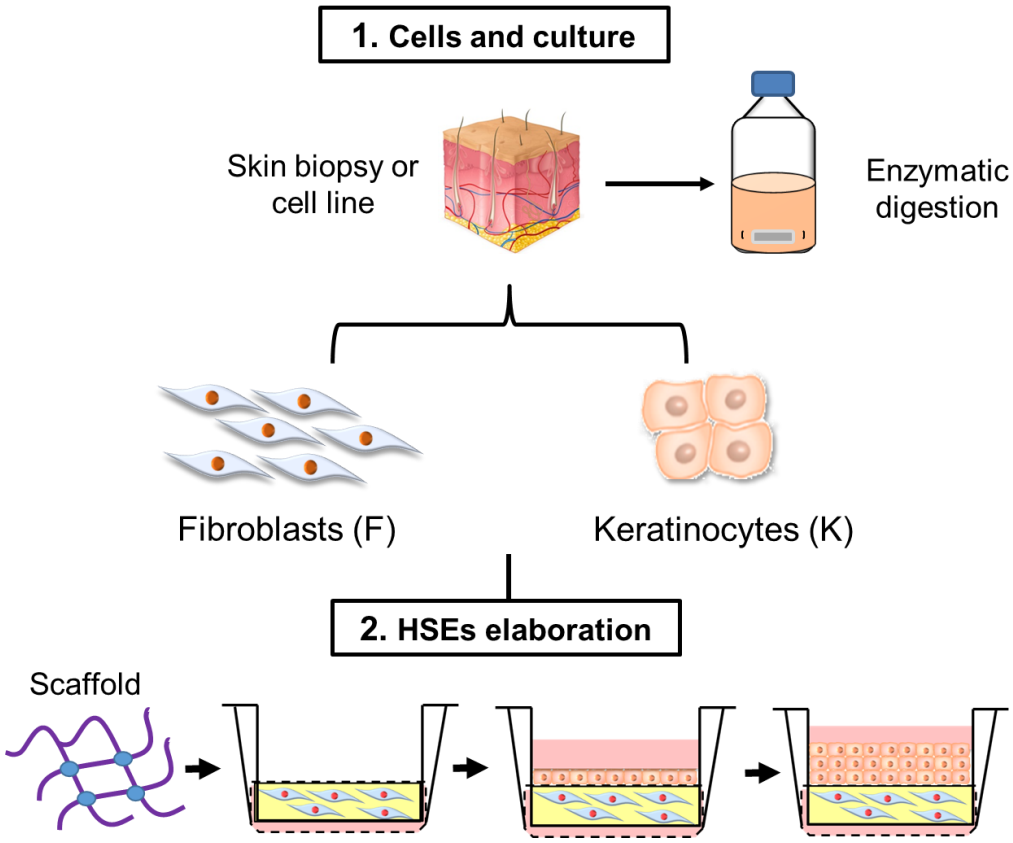

Figure 3. General process for the generation of human skin equivalents.

Some commercially available human skin equivalents for *in vitro* testing are listed in Table 2.

Table 2. Commercially available human skin substitutes (epidermal composites).

Product	Company	Description	Ref.
Epiderm™	MatTek	Neonatal human-derived fibroblasts and keratinocytes cocultured to form a multi-layered, highly differentiated model of the human dermis and epidermis.	83
StrataTest	StrataTech	Full thickness skin model composed of Normal Immortal Keratinocytes, and dermal fibroblasts embedded in type I collagen.	84
RealSkin	EPISKIN/ L'Oréal	3D skin model made of keratinocytes and fibroblasts	81
Advanced Skin Test 2000 (AST2000)	CellSystems	Dermal equivalent with embedded fibroblasts, a differentiated epidermal layer of keratinocytes on top, and a functional basal lamina.	85,81
Apligraf	Organogenesis	Skin substitute composed of an epidermal layer containing keratinocytes and stem cells, and a dermal layer containing fibroblasts.	86
Full-thickness model	Michniak group	Fibroblasts seeded in a bovine collagen matrix and epidermal layer of keratinocytes on top.	81
SkinEthic™ RHE-LC	EPISKIN	Co-culture of keratinocytes, melanocytes and CD34+ hematopoietic progenitor cells, which differentiate into langerhans cells, onto de-epidermized acellular human dermis.	87
Permaderm™ (Cincinnati Shriners Skin Substitute)	Regeninic Inc, USA	Autologous fibroblasts and keratinocytes in culture with bovine collagen and GAG substrates.	69
Tiscover™ (A-Skin)	Advanced Tissue Medicinal Product, The Netherlands	Self-assembled autologous skin substitute with fibroblasts and keratinocytes.	88

TissueTech Autograft System (Laserskin and Hyalograft 3D)	Fidia Advanced Biopolymers, Italy	Hyaluronic acid with cultured autologous keratinocytes and fibroblasts.	88
Dermal-epidermal organotypic culture	Tissue Engineering and Cell Therapy Group	Human fibroblasts embedded in a fibrin matrix and human keratinocytes cultured on top forming a differentiated epidermis.	82,89

GENE-MODIFIED HSEs

The field of tissue engineering has made significant progress through the application of gene delivery. Gene therapy can be used in order to enhance existing cellular activities in tissue substitutes.[90] This is achieved by genetically modifying the cells grown in the scaffolds or by transferring genes to the injury site. In skin tissue engineering, epidermal keratinocytes and/or dermal fibroblasts can be genetically modified with viral or non-viral vectors.[91,92] These genetically modified cells are then used to produce three-dimensional HSEs, acting as "bioreactors" *in vivo* to produce and secrete proteins of interest.[93,91,94] For example, local delivery of microbial peptides could be used to treat skin wounds, prevent infectious processes, and allow better tissue regeneration.

Different methods for gene delivery

Although numerous studies have focused on gene therapy for multiple cutaneous diseases, some of these therapies have not advanced to the clinical phase due to their risk to patients. Recombinant viruses have been widely utilized as vectors for gene transfer. However, viral vectors have several disadvantages, including the immunogenicity of adenoviruses, the oncogenicity of retroviruses, and the generation of different mutagenic processes of some adeno-associated viruses.[95,96] In spite of this, some studies on gene-enhanced skin substitutes through the viral expression of innate antimicrobial peptides (e.g., cathelicidins and β-defensins) have been reported.[97-99] In these studies, cells that overexpressed the antimicrobial peptide of interest showed high activity against methicillin-resistant *S. aureus* and *P. aeruginosa*. These gene-transfer approaches still require careful safety and efficacy considerations before their clinical application.

Alternative strategies to address the limitations of viral-based expression systems have been established. Among these, non-viral vectors are receiving increasing attention for clinical application in gene therapy. [100,101] The major advantage of using these vectors is their bio-safety, in addition to other properties such as their low cost and ease of production. However, the main challenge in applying non-viral gene transfer is the relatively poor efficiency of transfecting host cells compared to viral methods.[102] This has led current research to focus on the development of ideal vector delivery systems.

To date, several types of non-viral vectors have been developed, which can be classified into two main categories: physical and chemical methods.[100] Physical methods use physical force to counteract the membrane barrier of the cells, which facilitates the intracellular release of genetic material.[103] These physical methods include microinjection, ballistic DNA, electroporation or nucleofection, sonoporation, and photoporation.[100,104] One of the most reliable physical methods for delivering plasmid DNA to the skin is electroporation.[105,106] This technique is based on applying electrical pulses to the cellular membrane, which produces small pores through which plasmids can enter the cytoplasm and go to the cell nuclei, where the gene of interest is expressed. After plasmids enter the cells, the process can be reversed so that the pores in the cell membrane close rapidly, thus maintaining viability.[107] Chemical non-viral nucleic acid delivery systems are used more frequently than physical methods.[108] Generally,

they are nanomeric complexes, which are formed from the compaction of negatively charged nucleic acids by polycationic nanomeric particles. These systems include the lipoplexes (complex between a cationic liposome or micelle and nucleic acids), and the polyplexes (complex between a cationic polymer and nucleic acids).[109] Chemical systems have been shown to be very efficient to enter cells predominantly via endocytosis. Unlike viral systems, they are characterized by their low toxicity and antigenicity.[107]

Polyplexes have emerged as a promising alternative in gene therapy within the chemical carriers group due to their chemical diversity and potential for functionalization.[110] Polyethylenimine (PEI) has been defined as the gold standard polycationic transfectant, being one of the most positively charged polymers, which can be synthesized in both linear (LPEI) and branched (BPEI) forms.[102,111,112] One of the main advantages of PEI is that it forms toroidal particles, which are stable to aggregation under physiological buffer conditions. Furthermore, PEI also has a great buffering capacity due to the high number of amine groups, most of which are not protonated at physiological pH. [113-115] Additionally, they can integrate various cellular ligands to increase their specificity and favor intracellular trafficking.

Gene delivery into hydrogels- the potential of fibrin gels

In recent studies, local delivery of genetic material through hydrogel scaffolds using PEI polyplexes has been evaluated. Results showed that the polyplexes increased the release rates of the genetic material compared to uncomplexed material. Moreover, they decreased cellular cytotoxicity, which could favor the applicability of these complexes in tissue regeneration.[116,117]

Among the hydrogels that have been used successfully for protein delivery through the PEI polyplexes system, the fibrin gel has attracted increasing attention for skin engineering applications because of its physiological function during wound healing.[118-120] Fibrin exhibits high biocompatibility, promotes cell attachment, and can be easily degraded by cells.[121] Additionally, fibrin gel can closely mimic the natural blood-clotting process and can self-assemble into a stable 3D network that can stretch up to three times their relaxed length before breaking.[122] However, fibrin gel as an ideal scaffold has two drawbacks: gel shrinkage during cell culture and low mechanical stiffness, which

could be explained due to fibrinolytic degradation.[119] Furthermore, the fibrin networks form a gel with high porosity, which despite being an important aspect for the exchange of nutrients and waste products, also implies that the fibers expel large amounts of water and collapse when submitted to deformation stress.[123]

These limitations can be solved by combining fibrin gel as a cell carrier with high porosity biodegradable scaffolds. In this regard, agarose has been proposed as a potential candidate for adding more stiffness and durability to the fibrin gel.[124] Agarose is a linear polysaccharide derived from agar, a natural product of red seaweed. It has been used for multiple tissue engineering applications due to their resemblance to the native ECM, and mechanical properties easy to adjust by concentration.[124,125] Agarose and fibrin form a hydrogel independently from one another.[126] While the fibrin clot can be produced by covalent crosslinking through factor XIII, agarose is a thermo-reversible self-assembling polymer that forms gels through non-covalent interactions.

Previous studies have already demonstrated the potential of fibrin-agarose-based hydrogels as substitutes for tissue engineering.[127,128] It was found that these hydrogels could resemble the ECM of many tissues such as the cornea,[129] skin,[130] oral mucosa,[131] and cartilage.[132] The ECM is a fundamental component of tissues, consisting of an organized 3D network of proteins and proteoglycans, which surround cells and provide them with a fundamental microenvironment for multiple cellular processes necessary for tissue homeostasis and regeneration.[133,134] In addition to the improved biological properties, fibrin-agarose-based hydrogels proved to be more versatile than fibrin gels alone. Their biomechanical properties can be modified by adjusting the agarose content.[118,135]

Thesis aims and outline

Although different treatments for skin wounds are currently available, infections caused by microorganisms resistant to conventional antimicrobial therapies continue to be a challenge for the recovery of patients.

The aim of the research described in this thesis is the development and validation of a robust fibrin-based human skin equivalent (HSE) with over-expression of the antimicrobial peptide LL-37. To this end, human keratinocytes and fibroblasts were genetically modified with a non-viral vector containing the precursor gene leading to LL-37 production.

The different aspects of the research towards this aim are as follows:

1. Determining the efficiency of transfection and the viability of transfected cells by comparing two non-viral transfection systems in primary cells: polyplexes and nucleofection (Chapter 2).
2. Determining the conditions for producing polyethylenimine polyplexes for non-viral transfection of keratinocytes and fibroblasts (Chapter 3).
3. Producing and evaluating HSEs containing transfected human primary fibroblasts and keratinocytes (Chapter 4).
4. Optimizing the mechanical characteristics of the fibrin-based HSEs while preserving an optimal niche for the cells (Chapter 5).

The results obtained in these studies are discussed in Chapter 6.

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Chapter 2

Polyplex System Versus Nucleofection for Human Skin Cell Transfection and Effect of Internal Ribosome Entry Site Sequence

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Tissue Engineering Part C Methods. 2018; doi:10.1089/ten.tec.2017.0435

ABSTRACT

Nonviral transfection has important implications on gene therapy because of its safety. In particular, polyfection and nucleofection are two widely used systems for nonviral gene delivery. Their potential depends on the transfection efficiency achieved, which is influenced in turn by the type of cells transfected and by the plasmid that carries the gene of interest. The efficiency of transfection by polyfection or nucleofection in human fibroblasts and keratinocytes was evaluated in this study. Transfections were performed with plasmids containing a gene of interest (human cathelicidin antimicrobial peptide) and two reporter genes (red or green fluorescent protein) that included or not an internal ribosome entry site (IRES). The efficiency was measured by flow cytometry in terms of percentage of cells expressing the reporter gene; viability of transfected cells was also evaluated. It was found that nucleofection was more efficient than polyplexes for transfecting fibroblasts and keratinocytes. Regarding the viability of fibroblasts after transfection, values were high in both systems. In contrast, keratinocytes were more sensitive to nucleofection. It was also noted that both types of cells decreased reporter gene expression when IRES sequence was located upstream of the reporter gene, suggesting a negative effect on the expression of this gene. These results confirm that the transfection efficiency depends on the type of cells and the system used.

INTRODUCTION

Genetic modification of skin cells (mainly fibroblasts and keratinocytes) is an important field of study because it could lead to more functional skin grafts. For example, the overexpression of molecules such as antimicrobial peptides in keratinocytes could help in early contamination and infection with multiresistant microorganisms.[1,2] In particular, LL-37 peptide has been described not only because of its antibiotic properties but also by its signaling molecule nature.[3] As well, the inclusion of angiogenic growth factors could help to avoid rejection of graft owing to nonintegration and nonefficient vascularization.[4]

Although cell transfection has been widely studied in the literature, it is necessary to empirically determine the technical requirements for a given cell culture type.[7] In this article, the focus is on the transfection of fibroblast and keratinocytes cell types.

Many researchers are currently developing non-viral transfection systems which have been regarded as safer than systems based on viruses. Two of the more widely adopted are polyfection and nucleofection.[6] On the one hand, polyfection refers to the transfer of genes into cells through cationic polymers, mainly linear or branched polyethylenimine (PEI). The PEI has a high number of surface primary amino groups that interact with DNA, forming polyplex particles protecting DNA from degradation before reaching the nucleus. These kinds of systems are the most simple and economic ones, and their use has become popular due to their low cytotoxicity and relative effectiveness *in vitro*.[7] On the other hand, nucleofection is based on the transient hydrophilic pores formation in the cell membrane. Nucleofection has been described as a more efficient with respect to chemical systems, and any mammalian cell can be transfected by nucleofection.

An important step in cell modification is achieving high efficiencies in transfection. That is why efficiency is a key factor in comparison of different transfection methods. Expression of reporter genes introduced in plasmid vectors is used for measuring the transfection efficiency and could allow tracking of transfected cells. However, some products of gene expression are metabolized extracellularly (e.g., *CAMP* gene expression). In these cases it is necessary to separate the reporter gene expression from the interest product. Introducing internal ribosome entry sites (IRES) sequences in the vectors enables the expression of two genes controlled by one single promoter in target cells.[8] In

bicistronic vectors containing the IRES sequence, the first gene (upstream IRES) is translated in a cap-dependent manner and the second one (downstream IRES) in an IRES-dependent manner. These systems have been widely used for *in vitro* and *in vivo* applications since their discovery, although it has been reported that the expression levels of genes located downstream of IRES sequence are lower than those levels expressed by genes located upstream.[9] This means that low levels of a reporter gene located downstream of the IRES sequence can result in an underestimation in the expression of the gene of interest, the latter is generally located upstream of the IRES sequence and with a translation cap-dependent,[10] which in the translation, serves as a "molecular tag" that marks the spot where the 40S ribosomal subunit is to be recruited.

In this study, primary human skin cells, fibroblasts, and keratinocytes had been genetically modified by polyplexes and nucleofection with a plasmid vector containing a gene of interest (CAMP) and a reporter gene (red fluorescent protein [RFP] or green fluorescent protein [GFP]). IRES sequences were included in the CAMP plasmid backbones in an upstream position with respect to the reporter gene. The efficiency of transfection and the viability of transfected cells were compared:

- Between the two different types of cells (fibroblasts and keratinocytes).
- Between the two transfection systems: polyfection and nucleofection. In all cases, three nucleofection commercial kits were compared: Mirus from Ingenio and L, V from Lonza.
- Between the transfection systems with and without the IRES sequence.

The expression of *CAMP* gene in fibroblast was further evaluated using PCR analysis. The reporter genes were expressed by both types of skin cells even when IRES sequence was included in the plasmid vectors. An interesting application of these findings could be the production of autologous skin three-dimensional organotypic cultures based on cells with the aforementioned modifications. These enhanced organotypic could be more efficient in the treatment of skin wounds.

METHODS

Plasmidic DNA Preparation

Lentivector with bicistronic IRES-tRFP (destination vector PS100080 from OriGene) allows both the reporter gene and the gene of interest expression. PrecisionShuttle system was used to introduce the CAMP sequence coding to the LL-37 expression; this sequence was obtained from a pCMV6 vector (entry vector RC208872 from OriGene). CAMP sequence was verified into PS100080 (Macrogen Inc.). From now on, the modified plasmid will be called PS100080-CAMP vector. All plasmids were amplified into competent *Escherichia coli* and purifications were performed by using a plasmid maxi kit (Quiagen EndoFree®). PmaxGFP® (Lonza) was used as the non-IRES-containing plasmid control.

Primary human fibroblasts and keratinocytes cell culture

With prior informed consent, approved by the Bioethics Committee of the Faculty of Medicine from University of Antioquia, primary human keratinocytes and fibroblasts were isolated from remaining skin obtained from surgical procedures. Collection, expansion and cryopreservation of keratinocytes and fibroblasts were performed by explants[11] and/or enzymatic digestion of the skin samples, according to the protocols established by the Group of Tissue Engineering and Cellular Therapy. Skin samples were mechanically fragmented and enzymatically digested by using trypsin/ ethylenediaminetetraacetic acid (Sigma-Aldrich). Cell pull obtained was cultured in two different media to obtain fibroblasts or keratinocytes.[11] Cells subcultured between one and five times were used for all experiments.

Polyplexes system transfection: LPP (linear PEI poliplexes)

The transfections were performed by using Linear PEI (LPEI 25 kDa, Polysciences). The procedure was based on a protocol described by Hsu and Uludag.[7] Some changes were introduced; the medium of transfection was the same used to culture the keratinocytes without antibiotic. In brief, polyplexes at nitrogen/phosphate ratio = 19 (mol from units of LPEI/mol from nucleotides of PS100080-*CAMP* or pmaxGFP) were formed in a buffered saline solution before putting them in contact with the cells. Fibroblasts or keratinocytes were subcultured 24 h before transfection and seeded at a density of 3 $x10^4$ cells/cm^2. The replacement of the cell medium was performed with the polyplexes (containing 1 μg of the plasmid) previously diluted in the medium of

transfection. Polyplexes were allowed to be in contact with the cells during 6 h, after cell medium was replaced by complete fresh medium and incubated overnight until cytometry analysis.

Nucleofection system transfection

Transfection by nucleofection of primary human fibroblasts and keratinocytes were performed by using the Nucleofector™ II/2b (Amaxa; Lonza). Different kits of electroporation were evaluated: Cell Line Nucleofector® Kit L and V (Lonza) and Ingenio® Electroporation Kit (Mirus). For fibroblast and keratinocytes programs, U-30 and T-018 were used, respectively. Protocols stablished by each kit were followed: electroporation volumes of 100 μl were used to transfect 1.5 x 10^6 cells with 2.5 μg of the plasmid (PS100080-*CAMP* or pmaxGFP), recommendations of the normal protocols for nucleofection.

Flow cytometry

Measures were performed in a BD LSRFortessa™ cytometer. Agglomerated cells were excluded by adjusting cytometer voltage. Ten thousand events were counted by each measurement. Analysis of results obtained by flow cytometry was done so that the population of autofluorescent cells was excluded from the positive population by the expression of the reporter gene (RFP or GFP). For this, a diagram was plotted with fibroblasts or keratinocytes that were not transfected (negative control). The type of diagram used was a ''pseudocolor'': Comp-FITC versus Comp-PE-A. Cells that were above the fluorescence intensity of 10^3 and simultaneously near to the line at 45° were considered autofluorescent.

Fibroblasts RNA isolation, cDNA synthesis and quantitative real-time PCR (polyplexes)

Total RNA from *in vitro* cultured human fibroblasts transfected with polyplexes was isolated with TRIzol reagent (Gibco). RNA integrity was assessed using agarose gel electrophoresis stained with ethidium bromide. cDNA was synthesized from 2 μg of total RNA by using the RetroScrip kit (Qiagen, Basel, Switzerland) according to the manufacturer's manual.

Quantitative real-time PCR was performed by using 2 μL of cDNA and 18 μL of SYBR Green PCR master mix (Thermo Fisher Scientific). *CAMP*- specific PCR primers were used to generate 100- bp fragments. All primers were synthesized by Macrogen (forward primer: 5′ CGCCAAAGCCTGTGAGCTT-3′;

reverse primer: 5′-TTCACCAGCCCGTCCTTCTTG-3′). A number of *CAMP* copies were obtained through the generation of a standard curve achieved with serial dilutions of PS100080-*CAMP* plasmid.

Statistical Analysis

Fibroblasts and keratinocytes were isolated from three different donors and triplicates of the transfections were made. Nonparametric tests were used. Kolmogorov-Smirnov test was used to compare the results coming from two independent samples: cells transfected by linear polyethylenimine polyplexes (LPP) and nucleofection. Kruskal-Wallis analysis of variance was used to compare the results coming from three independent samples of cells transfected by nucleofection using kits L, V, or Ingenio.

RESULTS

Nucleofection kits comparison: kits L, V, and Ingenio

Ingenio Electroporation Kit was evaluated in terms of transfection efficiency and compared with the L and V kits from Lonza, which are the recommended kits to transfect human skin cells by nucleofection. The comparison was performed using the pmaxGFP vector (Figure 1). Efficiency in all cases is defined as the ratio between cells expressing the reporter gene and the total number of cells. Both of these quantities were measured by flow cytometry.

On the one hand, when nucleofection was used in fibroblasts, results from the three kits show no significant differences between efficiency medians. All efficiencies were higher than 26% despite the fact that there were variations inside the samples, even for Ingenio Electroporation Kit. On the other hand, when nucleofection was used on keratinocytes, Ingenio Electroporation Kit had significantly lower efficiency than kits L and V. For keratinocytes, efficiencies of nucleofection performed with Ingenio Electroporation Kit were as low as 15.6%, while for kits L and V, efficiencies were as high as 44.4% and 55.3%, respectively. Therefore, data of three kits of nucleofection were included for fibroblasts, whereas for keratinocytes were not considered those obtained from Ingenio Electroporation Kit tests.

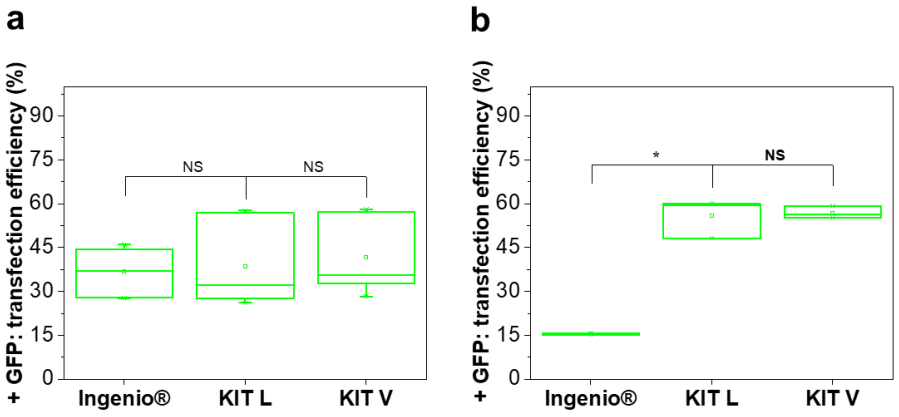

Figure 1. Nucleofection kit comparison. Transfection efficiency in nucleofected cells analyzed by flow cytometry at 24 h postnucleofection. (a) Fibroblasts and (b) keratinocytes. Kruskal–Wallis analysis of variance, n = 9, no significant differences (NS): p = 0.42, *p = 0.002. GFP, green fluorescent protein.

Nucleofection versus linear polyplexes: fibroblasts

Figure 2 shows representative images of fluorescence micrographs and flow cytometry analyses of fibroblasts transfected by nucleofection and polyplexes. In both systems there were transfected fibroblasts expressing the GFP but there were a notoriously higher number of green fibroblasts in nucleofection when compared with the polyplex system.

Flow cytometry measures confirmed the optical observations, and a quantitative analysis was performed (Figures 2 and 3).

Results of the quantitative analysis are shown as the median values of transfection efficiencies and viabilities of fibroblasts transfected by nucleofection and polyplex systems (Figure 3). Significant differences were found when both systems were compared. As expected, transfection efficiencies for nucleofection (median = 34.6%) were higher than the ones for the polyplexes (median = 14.8%). Regarding cytotoxicity, viabilities were under 50% for some samples when polyplexes were used, while viabilities were always higher than 68.7% when nucleofection was used.

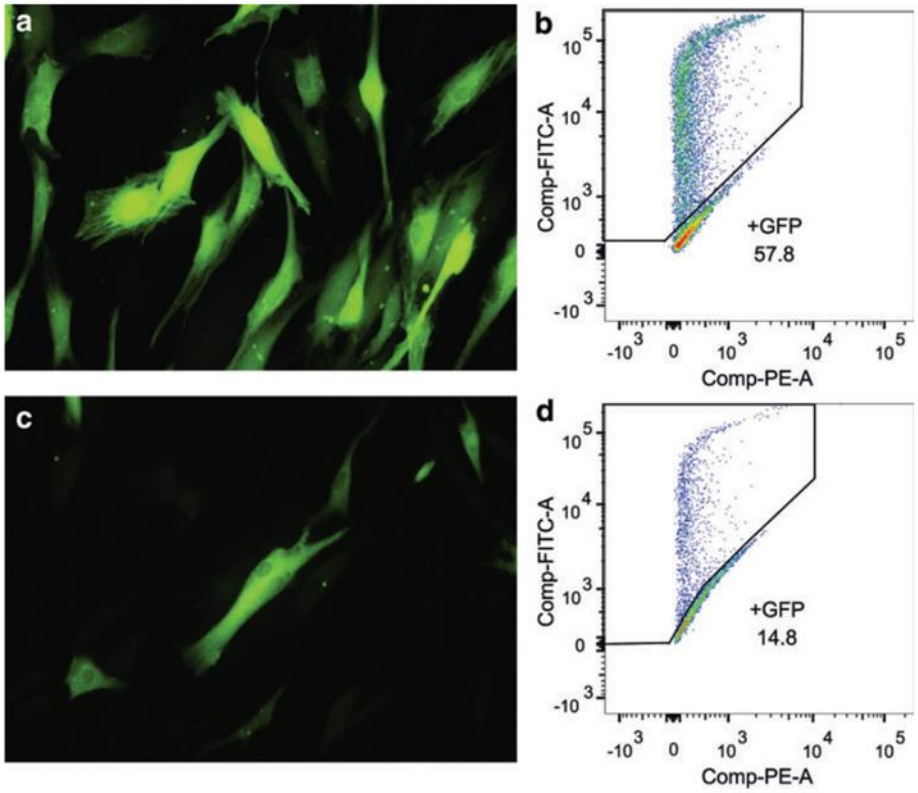

Figure 2. Nucleofection versus transfection with LPP in fibroblasts. (a, b) Nucleofection and (c, d) polyplexes. (a, c) Detection of the positive cells by fluorescence microscopy in fibroblast at 24 h post-transfection (40 X). (b, d) Quantification of transfection efficiency by flow cytometry. Transfection efficiency was up to 57.8% with nucleofection and 14.8% with polyplexes at 24 h post-transfection. LPP, linear polyplexes.

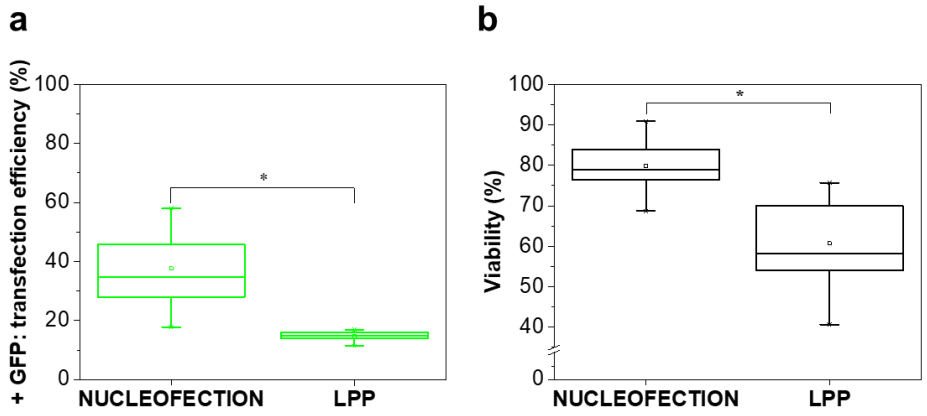

Figure 3. Fibroblasts. Nucleofection versus LPP. (a) Transfection efficiency and (b) cell viability analyzed by flow cytometry at 24 h post-transfection. The cell viability was determined using propidium iodide. K-S test n = 9, +GFP *p = 1.2 x 10^{-8}, viability *p = 0.0005. K-S, Kolmogorov– Smirnov.

Nucleofection versus linear polyplexes: keratinocytes

Figure 4 shows representative images of flow cytometry analyses and fluorescence micrographs of keratinocytes transfected by nucleofection and polyplexes. Similar to fibroblasts, in both systems, there were cells expressing the GFP and there were a higher number of keratinocytes expressing GFP with nucleofection. However, in contrast to fibroblasts, a high number of dead cells were observed with nucleofection.

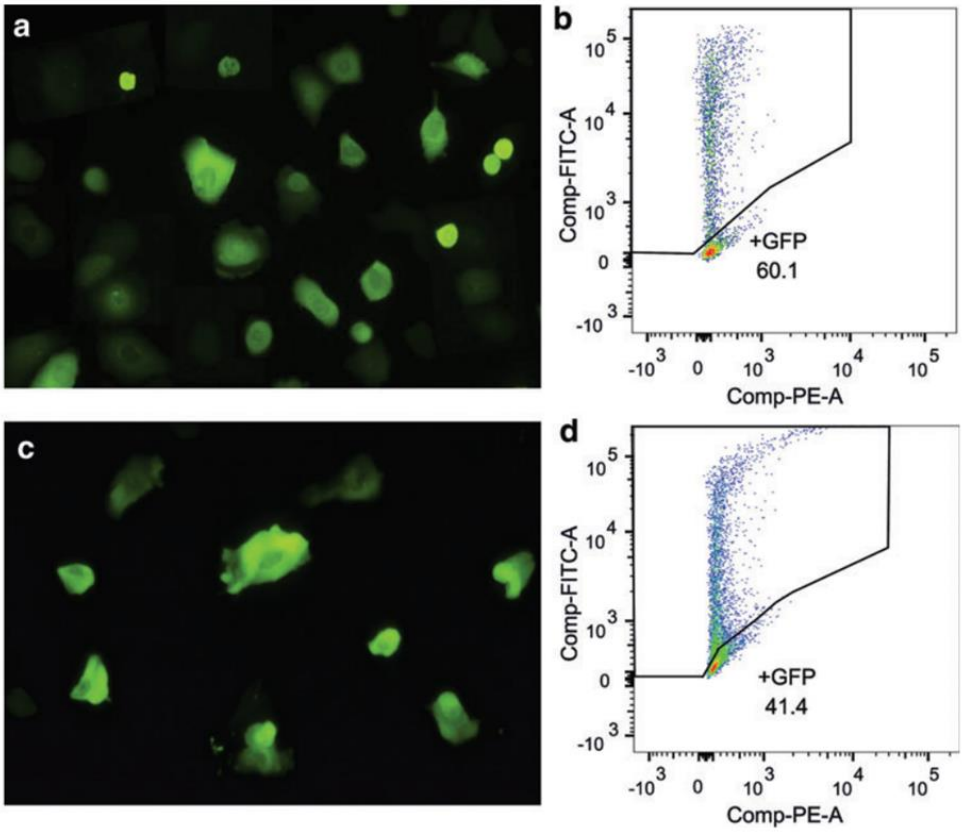

Figure 4. Nucleofection versus transfection with LPP in keratinocytes. (a, b) Nucleofection and (c, d) polyplexes: LPP. (a, c) Detection of the positive cells (+GFP) by fluorescence microscopy at 24 h post-transfection (40 X). (b, d) Quantification of transfection efficiency by flow cytometry. Transfection efficiency was up to 60.1% with nucleofection and 41.4% using LPP at 24 h post-transfection.

Quantitative results from flow cytometry are summarized in Figure 5. Efficiency of keratinocyte transfection were between 40% and 60% when nucleofection was performed, whereas values between 18% and 30% were achieved when polyplexes were used.

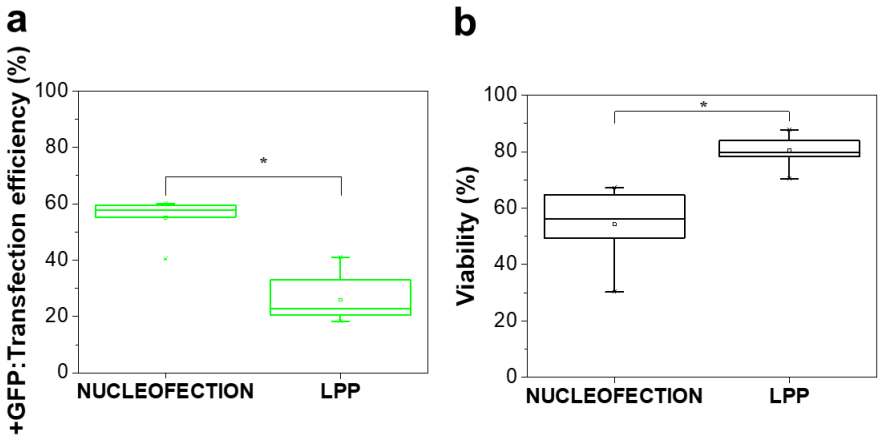

Figure 5. Keratinocytes. Nucleofection versus LPP. (a) Transfection efficiency and (b) cell viability analyzed by flow cytometry at 24 h post-transfection. The cell viability was determined using propidium iodide. K-S test n = 9, +GFP *p = 0.041, viability *p = 6.8 x 10^{-8}

Polyplex system: plasmid with IRES sequence versus no IRES sequence

We also evaluated the effect of an IRES sequence in the plasmid vector used to separately introduce both the gene of interest and the reporter gene in both types of cells. As mentioned in the ''Introduction'' section, it has been described in the literature that the expression levels of genes located downstream of an IRES sequence are lower than those of genes located upstream of them.[9] Since the reporter gene in our plasmid is downstream of the IRES sequence, we evaluated its effect on the expression of the reporter gene by transfecting fibroblasts and keratinocytes with polyplexes. This system showed lower transfection efficiencies in both kinds of cells measured through the expression of the reporter gene.

Figure 6 shows representative images of flow cytometry analysis and fluorescence micrographs of fibroblasts and keratinocytes transfected by polyplexes. The plasmid used contains the *CAMP* gene (interest) upstream the IRES sequence, and downstream of it is located the RFP gene (reporter). A small population of cells (both fibroblasts and keratinocytes) expressing the RFP were qualitatively observed (Figure 6(a) and 6(c)). Flow cytometry results confirmed the optical observations (Figure 6(b) and 6(d)).

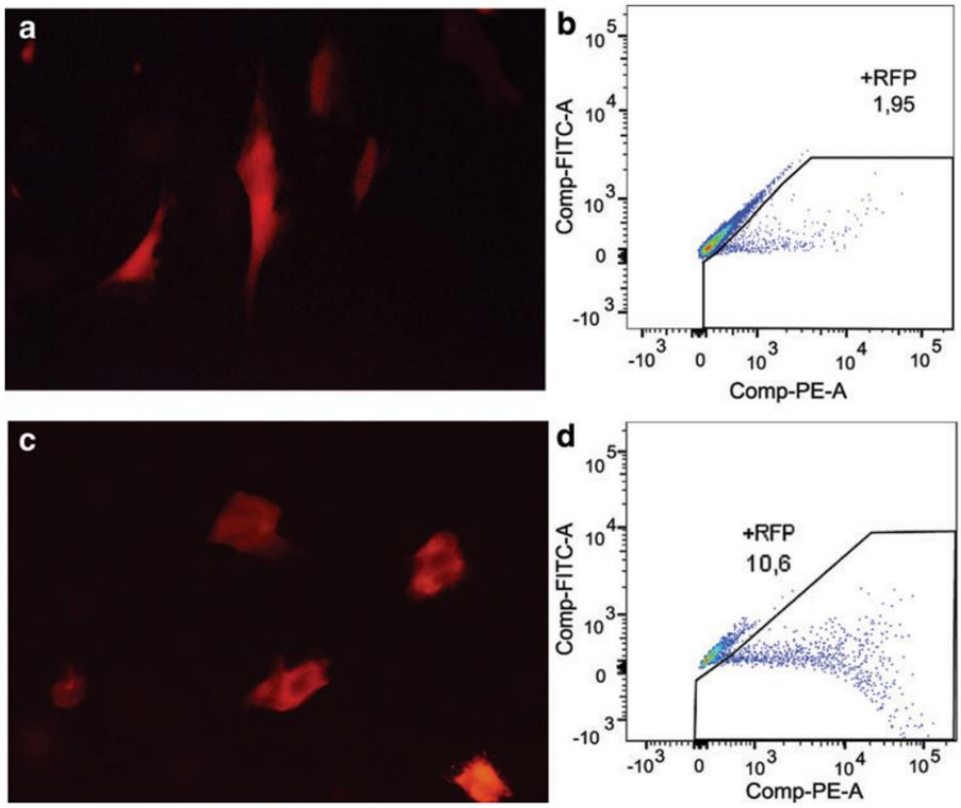

Figure 6. LPP transfection with a plasmid containing an IRES sequence. (a, b) Fibroblasts and (c, d) keratinocytes. Fluorescent optical micrographs (a, c) and flow cytometry (b, d), 40 X. The efficiency of transfection was quantified by flow cytometry 24 h post-transfection. IRES, internal ribosome entry site; RFP, red fluorescent protein.

Figure 7 shows the quantitative comparison between the transfection efficiencies and viabilities of fibroblasts and keratinocytes transfected by polyplexes with and without the IRES sequence upstream of the reporter gene (RFP or GFP, respectively).

Results showed significant differences between efficiency of transfection in fibroblasts and keratinocytes when a vector containing an IRES sequence was used in the transfection. Regarding the expression of the reporter gene, (1) for fibroblasts, a median efficiency of 1.95% was obtained when the plasmid containing IRES (+RFP cells) and a median efficiency of 14.8% when the plasmid without IRES (+GFP) were used, and (2) for keratinocytes, a similar trend was observed, median efficiency of 4.5% was obtained when the plasmid contained

IRES (+RFP cells) and a median efficiency of 22.8% when the plasmid without IRES (+GFP) was used.

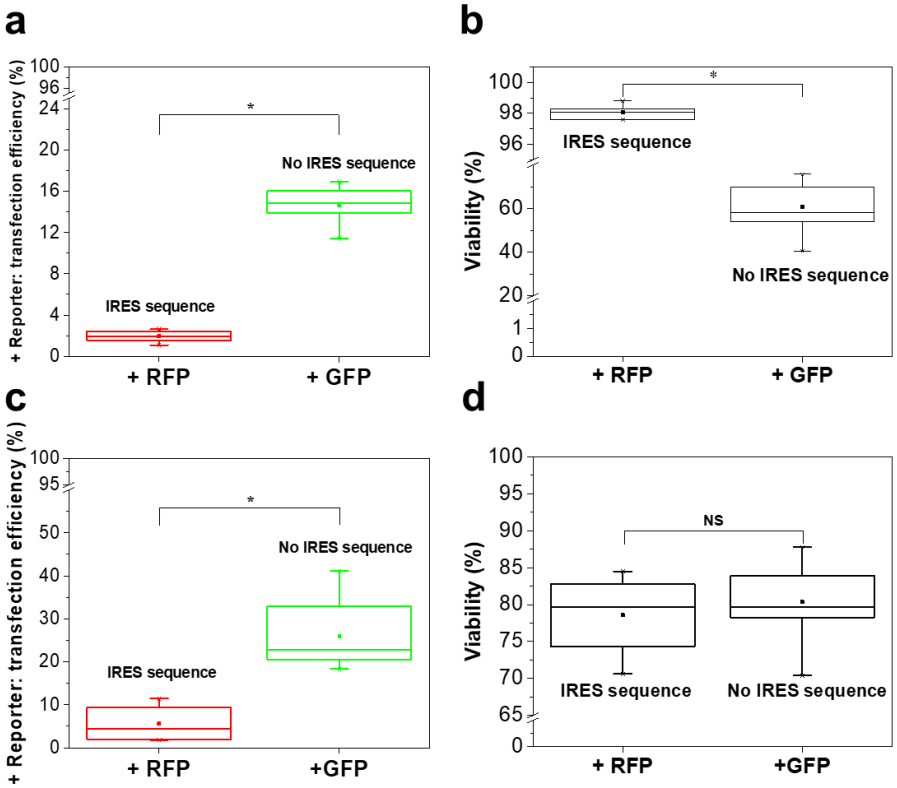

Figure 7. LPP transfection with a plasmid with or without IRES sequence: transfection efficiency and cell viability in (a, b) fibroblasts and (c, d) keratinocytes. The efficiency of transfection and viability were quantified by flow cytometry 24 h posttransfection. *p < 0.05.

Regarding cytotoxicity, significant differences were found for fibroblasts as follows: presence of IRES sequence increased the fibroblast viability from a median of 58.2% to 98.1%. Meanwhile for keratinocytes, no significant differences were observed with and without IRES sequence. Viabilities had a median of 79%.

Fibroblast CAMP expression (polyplexes)

Although the polyplex system exhibited low transfection efficiencies through reporter gene expression due to the use of IRES sequence (Figures 3 and 5), further PCR analysis allowed us to find that the expression of the gene of interest was, in fact, higher than in the controls (nontransfected cells). To confirm this hypothesis, we evaluated the expression of the *CAMP* gene in fibroblasts transfected with polyplexes, since it is known that these cells do not express the *CAMP* gene constitutively. Figure 8 shows the results of the *CAMP* gene-copy number quantification in transfected versus control cells (nontransfected fibroblasts). Overexpression of *CAMP* gene was observed at 24 and 48 h post-transfection.

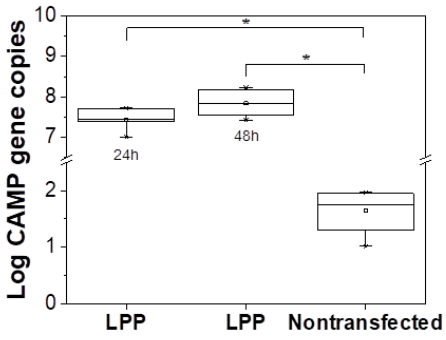

Figure 8. Quantification of *CAMP* gene expression in human fibroblasts. The cells were transfected with LPP and homogenized with TRIzol for the extraction of total RNA. The number of copies of the *CAMP* gene was quantified by real-time PCR system (LightCyclerr® 96; Roche) at 24 and 48 h post-transfection versus controls (nontransfected fibroblasts), K-S test, n = 3, *p < 0.05.

DISCUSSION

Nucleofection has been reported as an efficient transfection system for human cells from primary cultures.[12,13] In particular, L and V commercial kits from Lonza have been described as very good options in nucleofection of skin human cells (fibroblasts and keratinocytes). However, there exist other less expensive kits that could be used in the transfection of primary cell cultures. Ingenio Electroporation Kit is one of them, and in this study, it was compared with L and V kits from Lonza with respect to nucleofection of primary skin fibroblasts and keratinocytes. Fibroblast results have shown that all three kits were efficient in terms of transfection efficiencies. Obtained efficiencies (Figure 1(a)) were higher than other reported transfection systems applied to primary human fibroblasts.[7] In the comparison of nucleofection applied to primary keratinocytes with three different kits (Figure 1(b)), results showed a significant decrease in the nucleofection efficiency when Ingenio Electroporation Kit was used. These results could be explained due to the fact that keratinocytes are electrolyte-dependent cells for its differentiation and survival processes,[14] and therefore, these kinds of cells can be more sensitive to the buffers who mediate the electroporation (nucleofection). Kits L and V could contain a supplement that protects cells from electrolyte misbalance, and consequently, that could lead to better transfection efficiency.

In addition to nucleofection, polyplexes are one of the most currently studied systems to genetically modify primary cells in an efficient and economical way. Furthermore, it is well known that results in transfection also depend on the type of cells, so a polyplex system made with LPEI to transfect primary skin fibroblasts and keratinocytes was also evaluated. As expected, efficiency of nucleofections were higher than those of polyplexes for both types of cells (Table 1).

Table 1. LPP Transfection and Nucleofection in Human Fibroblasts and Keratinocytes.

# of cells /100 cells	Fibroblasts		Keratinocytes	
	LPP	Nucleofection ([a],[b])	LPP ([a])	Nucleofection ([b])
Alive (viability)	58	80	80	56
Expresing (efficiency)	9	28	18	31

(a) Higher transfection efficiencies. (b) Healthier cultures. LPP, linear polyplexes.

Regarding the cytotoxicity of transfection, it was found that viability of fibroblasts had a median of 58% and 80% with polyplexes and nucleofection, respectively (Table 1). Results show that nucleofection has higher transfection efficiencies (a) and healthier cultures (b) than polyplexes when applied to primary human skin fibroblasts, as indicated in Table 1.

Comparison between nucleofection and polyplex systems for keratinocytes transfection is not as simple as for fibroblasts. Again, when transfection efficiencies were compared, nucleofection system seems to be better than polyplexes (Table 1). However, even though there were more cells expressing the reporter gene with nucleofection, viability decreased significantly (Table 1). Keratinocytes could be more sensitive to nucleofection due to the fact that this type of cells has an active transport of different kinds of molecules. These molecules, such as lipids and calcium ions, are required for the keratinocyte´s normal growth and differentiation processes.[15] In the case that modified skin cells are to be used in tissue engineering, obtaining suitable autologous grafts from healthy cultures is the main goal for tissue regeneration. In particular, skin equivalents built with keratinocytes expressing antimicrobial peptides could have a greater added value. Nevertheless, it is still necessary to have healthy keratinocytes that can differentiate, and form stratified epithelia to obtain similar equivalents to healthy skin. Therefore, polyplexes could be an appealing system to preserve the viability of the keratinocytes (a) despite their apparent lower efficiency.

In addition to high transfection efficiency and low cytotoxicity in some cases, one needs to obtain the molecule that is expressed from the gene of interest separated from the molecule that is expressed from reporter gene. For example, when the product of interest is a peptide, the reporter molecule can interfere with the peptide activity because peptides are short-sized molecules, and their function depend on their tridimensional structure. Another issue that is related to

transfection tracking during cell culture is that if the product of interest is extracellular and the reporter protein is attached to the product, the reporter cannot be registered during cell culture. As mentioned before, IRES sequence enables separately and simultaneously expression of two genes controlled by one single promoter in target cells.

In this article we have compared two plasmids, both plasmids have a CMV promoter upstream of the reporter genes: RFP and GFP. However, only the RFP plasmid contains the IRES sequence upstream the RFP gene. Even though CMV is a strong promoter in many types of cells, [16] results showed that when IRES sequence is driving the expression of the reporter, it significantly decreases the expression in both types of cells (fibroblasts and keratinocytes) (Figure 7(a) and 7(c)). As showed by Mizuguchi et.al.,[10] expression of an IRES-dependent second gene decreases (in most cases between 20% and 50%) with respect of that of the first gene. In fact, the statistical analysis shows a significant difference between the efficiencies in terms of the reporter gene expression. This result could represent a problem when one needs to perform an estimation of the expression of the gene of interest through the quantification of cells expressing a reporter gene,[8] since the results of quantification of the copy number of the gene of interest (in this case the *CAMP* gene) using RT-qPCR, showed an overexpression in the transfected cells.

Overexpression of *CAMP* gene in fibroblasts transfected with polyplexes was shown to be higher when compared to controls (non-transfected fibroblasts). This result suggests that final delivery of the gene of interest is efficient enough to achieve a high number of gene copies although transfection efficiency measured by expression of the reporter gene is lower. In addition, *CAMP* gene expression continues to increase 48 hours after transfection with polyplexes (Figure 8), so this system could be attractive to ensure a sustained delivery of gene of interest in human fibroblasts.

Finally, the results showed that viability values were always higher than 70% when IRES sequence was included in the plasmid. For the fibroblasts, it was observed a statistically significant difference in viabilities with and without IRES sequence (Figure 7(b)). This can be due to the fact that a lower expression of the reporter gene reduces the cytotoxicity of the reporter gene (RFP). Viability of keratinocytes showed no significant differences (Figure 7(d)) and in both cases (with and without IRES sequence) viability was high. As discussed before, polyplexes can

be more useful in keratinocytes to preserve the viability of the cells when they are transfected.

CONCLUSION

Results showed that the three kits (L and V from Lonza and Ingenio from Mirus) were efficient in the nucleofection of fibroblasts, while only the L and V kits from Lonza were efficient in the nucleofection of keratinocytes. Nucleofection proved to be more transfection efficient in fibroblasts than polyplexes, but cell viability is best maintained in keratinocytes when a polyplex system is used.

Transfection with the presence of an IRES sequence seems to affect the expression of genes that are downstream of the sequence. Nevertheless, this effect is not necessarily negative, since it allows both (1) the confirmation of the transfection by means of a reporter and (2) the reduction of its expression, consequently decreasing the cytotoxicity of the transfection.

ACKNOWLEDGMENTS

The authors acknowledge IPS Universitaria (University of Antioquia) for allowing them to use their facilities and for the obtaining of skin biopsies. This work was financed by Colciencias code number 111556933571 CTO 581-2013. M.I.P.V. is a fellow from Colciencias National Doctoral program, code 727-2015.

DISCLOSURE STATEMENT

No competing financial interests exist.

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Chapter 3

Polyplex System to Enhance the LL-37 Antimicrobial Peptide Expression in Human Skin Cells

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Tissue Engineering Part A. 2020; doi: 10.1089/ten.tea.2019.0196

ABSTRACT

Inefficient autologous tissue recovery in diverse skin injuries increases the susceptibility of patients to infections caused by multiresistant microorganisms, resulting in a high mortality rate. Nonviral transfection is an attractive alternative for these patients, where genetically modified cells incorporated in skin substitutes could release additional antimicrobial agents into the native skin. In this work, we have modulated the conditions of using a nonviral system for transfection of primary human keratinocytes and fibroblasts, consisting of a polymer/plasmid DNA complex called polyplex and its effects on the expression of LL-37 antimicrobial peptide. Linear and branched polyethylenimine polymers in different weight concentrations were varied for evaluating the formation and colloidal characteristics of the polyplexes. The PEI/pDNA polyplexes with 19 Nitrogen/ Phosphate ratio, are nanometric particles (400 and 250 nm with lineal and branched polyethylenimine, respectively) exhibiting positive surface charge (+30 ± 2 mV). Both kinds of polyplexes allowed the expression of a reporter gene and increased the human cathelicidin antimicrobial peptide gene expression in transfected keratinocytes and fibroblasts; however, greater cytotoxicity was observed when polyplexes formed with branched PEI were used. Moreover, cell culture supernatants from transfected cells with linear polyethylenimine/pDNA polyplexes showed enhanced antimicrobial activity (decrease of bacterial growth in 95.8%) against a *Staphylococcus aureus* strain *in vitro*. The study of the polyethylenimine/pDNA polyplexes formation allowed to develop an improved transfection strategy of skin cells, promoting the production of LL-37 antimicrobial peptide.

INTRODUCTION

Skin wounds increase the susceptibility of patients to infections caused by multiresistant microorganisms, resulting in a high mortality rate when inefficient autologous tissue recovery is provided on time.[1,2] Genetic modification of skin cells has become an important field of study, because it could lead to the construction of more functional skin grafts, through the overexpression of antimicrobial peptides that would prevent early wound contamination and infection with bacteria.[3,4]

Although most gene delivery approaches so far have involved viral vectors, nonviral systems are receiving increasing attention as a more attractive alternative due to their safety, easy manufacturing, and low cost.[5,6] Currently, polyplexes (complex of a polymer and DNA) are one of the most studied nonviral systems to genetically modify primary cells in an efficient and economical way.[7–10] Specifically, polyethylenimine/plasmid DNA (PEI/pDNA) polyplexes pose a safer method of gene transfection compared with viral vectors. It has been described that to exert their function they have to cross several cellular barriers, including the plasma, endosomal, and nuclear membranes, with the endosomal escape being a critical aspect to release the cargo.[11] On the other hand, it is known that the transfection efficiency of polyplexes depends on the cell type, in addition to the size and structure of the polymer.[12,13]

PEI is available in a variety of molecular weights and structures, including linear PEI (LPEI) and branched PEI (BPEI). While both types of PEIs have achieved excellent transfection efficiencies in a wide range of cell lines, discrepancies in transfection abilities have been reported both *in vitro* and *in vivo*,[14,15] being necessary to determine the technical requirements to develop a transfection strategy for a given cell type.[16]

In this study, we compared the transfection of human skin cells (primary fibroblasts and keratinocytes) using LPEI and BPEI polyplexes, to obtain the overexpression of the human cathelicidin antimicrobial peptide (*CAMP*) gene, which encodes the production of the LL-37 peptide, whose antimicrobial properties in vivo have been widely shown for a number of Gram-positive and Gram-negative bacterial strains, as well as on the yeast *Candida albicans* and virus like herpes simplex.[17–22]

The *CAMP* gene expression was confirmed in keratinocytes and fibroblasts using both LPEI and BPEI polyplexes. Also, the cell culture supernatants of transfected cells with LPEI polyplexes exhibited antimicrobial activity against *Staphylococcus aureus* strain. These modified dermoepidermal cells could be used to produce temporary skin substitutes, which may allow to increase LL-37 peptide cell excretion, promoting an additional antimicrobial activity in the skin wound.

MATERIALS AND METHODS

Plasmids preparation (pDNA)

As a separate part of the PrecisionShuttle system, Lenti vector with bicistronic IRES-tRFP (destination vector PS100080 from OriGene) was used to express both the reporter gene (red fluorescent protein [RFP]) and the gene of interest (*CAMP*) that encodes the antimicrobial peptide LL-37. The *CAMP* sequence was obtained from a pCMV6 vector (entry vector RC208872 from OriGene), and the sequence integrity into PS100080 was confirmed by Macrogen, Inc. The modified PS100080-*CAMP* DNA plasmid (pDNA) was purified using a plasmid maxi kit (QuiagenEndoFree®). The concentration and integrity of the pDNA were determined using the NanoDrop 1000 Spectrophotometer (Thermo Scientific®, New Hampshire) and agarose gel electrophoresis, respectively.

PEI/pDNA polyplexes formation

Polyplexes were made by mixing 1μg/mL of purified pDNA (PS100080-*CAMP*) with different weights of LPEI or BPEI (LPEI, 25kDa; Polysciences, Inc. and BPEI, 25kDa; Sigma-Aldrich) in 4-(2-Hydroxyethyl) piperazine-1-ethane-sulfonic acid (HEPES)-buffered saline (150mM NaCl, 20mM HEPES, pH 7.4), and vortexed briefly and incubated at room temperature for 10min. The LPEI/pDNA and BPEI/pDNA polyplexes had different N/P molar ratios (nitrogen from PEI to phosphate from DNA) between 0 and 30. The binding of DNA to PEI to form polyplexes was evaluated by quantifying the amount of free DNA in the solution, by using the PicoGreen dsDNA assay (Invitrogen®). One hundred microliters of this reagent were added to 100μL of polyplexes sample, and the fluorescence was measured with a fluorometer (JASCO, FP-750) set at 480 and 520nm for excitation and emission, respectively, and it was converted to the corresponding DNA concentration using the following equation of the calibration curve (Equation 1):

$$\text{Free pDNA (ng/mL)} = 0.22802 + 0.04899\,x \quad [1]$$

where x indicates the emission intensities from uncomplexed standard DNA (Lambda DNA).

The PEI/pDNA polyplexes formation efficiency (PEI/ $pDNA_{FE}$) was expressed according to Equation 2:

$$PEI/pDNA_{FE} = (\text{Initial pDNA} - \text{Free pDNA})/\ (\text{Initial pDNA}) \times 100 \quad [2]$$

Size and zeta potential of PEI/pDNA polyplexes

The LPEI/pDNA and BPEI/pDNA samples, prepared in 20mM HEPES - 150mM NaCl solution with N/P ratio between 0.5 and 30, were analyzed by dynamic light scattering (DLS; HoribaLB-550) at pH 7.4 and 25°C, with a sampling time of 150s. Average size values were calculated with the Stokes equation from three runs. Analog samples were prepared in water for measuring the zeta potential by capillary electrophoresis (Malvern Zetasizer Nano series) at 25°C.

Human keratinocytes and fibroblasts isolation and culture

After previous informed consent, human keratinocytes and fibroblasts were isolated from skin samples obtained from discarded material from surgeries performed at the IPS Universitaria. Samples were processed according to the protocols established by the Group of Tissue Engineering and Cellular Therapy.[23] In brief, after removing fat tissue, samples were mechanically fragmented into small pieces (0.5–1mm^2) in sterile conditions and placed in T25 culture flasks (Falcon™). Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher Scientific, USA) high glucose (4.5 g/l), supplemented with 10 v/v% fetal bovine serum (FBS) and 1 v/v% penicillin–streptomycin (Gibco®) was added to obtain fibroblasts, and DMEM: HAM F12 (3:1) (Gibco) supplemented with 10 v/v% FBS (Gibco) was added to obtain keratinocytes.[23] Passages between 1 and 5 were used in all experiments.

Cytotoxicity and hemocompatibility assays

The thiazolyl blue tetrazolium bromide (MTT) assays were carried out to measure the cytotoxicity of the polyplexes for keratinocytes and fibroblasts. These cells were seeded into 24-well culture plates before transfection at a density of $3x10^4$ cells/cm^2 and incubated overnight. Then, LPEI/pDNA and BPEI/pDNA polyplexes with different N/P ratios were added and incubated with the cells at 37°C for 6h. The cells were then washed with phosphate-buffered saline (PBS, pH 7.4, 150mM), followed by the addition of 0.5 mL/well of MTT solution (1mg/mL in PBS). After incubation at 37°C for 2h, the MTT was removed from the well, and 1mL of isopropanol was added and incubated for 2h in continuous agitation (100rpm) at room temperature to dissolve any formazan crystals. The absorbance of this solution was measured at 570nm using a microplate reader (EPOCH BioTek), and the survival rate was calculated as percentage of control (untransfected cells) using Equation 3:

$$\frac{A_{570}\ of\ transfected\ cells - A_{570}\ of\ blank}{A_{570} of\ untransfected\ cells - A_{570}\ of\ blank}\ X\ 100 \quad [3]$$

For the hemocompatibility assay, guinea pig red blood cells (RBCs) suspension was obtained as a gift from Dr Y.C. Bhide (Medical Microbiology and Infection Prevention, University Medical Center Groningen, The Netherlands). After centrifugation for 10min at 2000 g at 4°C, the collected RBCs were washed with PBS (pH 7.4) until the supernatant was colorless. Fresh LPEI/pDNA and BPEI/pDNA polyplexes were prepared in 20mM HEPES -150mM NaCl solution at various N/P ratios. RBCs were added to obtain a final concentration of 8% (v/v) and incubated at 37°C for 1h. The mixture was centrifuged at 2000 g for 15min, and the absorbance of the supernatant was measured at 460nm to determine the amount of hemoglobin released. The HEPES– NaCl solution and 0.1% Triton X-100 were used as negative and positive controls, respectively. The hemolysis rate (%) was calculated by the following Equation 4:

$$\text{HR}\ (\%) = \frac{(OD_{sample} - OD_{negative\ control})}{(OD_{positive\ control} - OD_{negative\ control})}\ X\ 100 \quad [4]$$

In vitro cell transfection

Keratinocytes and fibroblasts were seeded 24h before transfection into 24-well plates with $3x10^4$ cells/cm^2 in 0.5mL EpiLife (Invitrogen™) or DMEM with 10% FBS (Gibco), respectively. The transfection procedure was performed following the protocol described by Hsu and Uludag.[24] In brief, 50μL of LPEI/pDNA or BPEI/pDNA polyplexes with N/P=19 (6.3 μg PEI per 2.5 μg pDNA, to maintain a PEI/pDNA ratio=2.5 w/w) were mixed with 0.5mL DMEM: HAM F12 (3:1) low in serum (1% FBS) without antibiotics, at 37°C, and added to the cells. Then, the plate was centrifuged at 210 g for 5min, at 25° C. After 6h incubation, transfection medium was replaced with fresh culture medium (DMEM: HAM F12 [10% FBS]).

Reporter gene expression

To quantify the transfection efficiency, keratinocytes and fibroblasts were trypsinized and washed in NaCl 1% SBF 24h post-transfection. After centrifugation at 1500rpm for 15min, the cells were stained by 7-Amino Actinomycin D (7-AAD; Sigma-Aldrich) in 0.3 mL PBS to select for live cells. A BD LSRFortessa™ flow cytometer was used to count the number of transfected cells by detecting the red fluorescence of RFP with nontransfected cells as the negative control. Ten thousand events were analyzed. FlowJo 10 software was used for data analysis, and the transfection efficiency was calculated as the percentage of positive cells to total cells. The RFP expression in cells was also verified by fluorescence microscopy.

RNA isolation, cDNA synthesis, and quantitative real-time polymerase chain reaction

Twenty-four hours after transfection, total RNA of keratinocytes and fibroblasts was purified using TRIzol™ Reagent (Invitrogen) according to the manufacturer's recommendations. A modification was introduced during the separation phase, doubling the amount of chloroform added to the samples to increase the reaction yield (0.4mL/ 1mL of TRIzol). RNA integrity was assessed using agarose gel electrophoresis stained with ethidium bromide. cDNA was synthesized from 2 μg of total RNA by using the RetroScrip kit (Qiagen) according to the manufacturer's manual. The copy number of the CAMP gene was quantified using the LightCycler® 96 real-time PCR system (Roche), with 2 μL of cDNA and 18 μL of SYBR Green PCR master mix (Invitrogen). CAMP-specific PCR primers were designed using the Primer3Plus software to generate 100-bp fragments; all primers

were synthesized by Macrogen, Inc. (forward primer: 5'-CGCCAAAGCCTGTGAGCTT-3'; reverse primer: 5'-TTCACCAGCCCGTCCTTCTTG-3'). Many CAMP copies were obtained through the generation of a standard curve achieved with serial dilutions of PS100080-CAMP plasmid.

***In vitro* antimicrobial activity assay**

The culture supernatant of modified keratinocytes and fibroblasts was collected 24h after transfection and stored at -20°C until use. The antimicrobial activity of these supernatants was evaluated against *S. aureus* strain ATCC 25923, cultured in the Laboratory of Microbiology, University of Antioquia. A volume of 50 µL of the supernatant was added to 50 µL of bacterial suspension with 10^4 CFU (colony forming units). This mixture was incubated for 1h at 37°C and 5% CO_2. Then, 10 µL were plated on blood agar plates and incubated at 37°C for 24h. Bacterial growth inhibition rate is given by Equation 5:

$$\frac{No.of\ colonies\ of\ the\ negative\ control - No.colonies\ in\ test\ sample}{No.colonies\ of\ the\ negative\ control} x\ 100 \quad [5]$$

Statistical analysis

Fibroblasts and keratinocytes were isolated from three different donors, and triplicates of the transfections were made. The Mann–Whitney U test was used to compare the results from two independent samples: cells transfected using polyplexes made with LPEI or BPEI. The one-way analysis of variance (ANOVA) was used to compare the means of cellular viabilities between the control cells (untransfected) and the transfected cells at different N/P ratios. A p-value of <0.05 was considered statistically significant.

RESULTS

Characterization of polyplexes

The formation of LPEI/pDNA and BPEI/pDNA polyplexes was followed by adding PicoGreen fluorophore, which react with the free pDNA leading to a fluorescence intensity decay. Then, the free pDNA was quantified, and the polyplexes formation efficiency calculated by Equation 2. The results show a decrease of the free pDNA when the N/P ratio was increased (an increase of PEI concentration) until reaching a plateau for both types of polymers (Figure 1).

The intersection between these two regions indicates the minimum critical concentration of PEI (MCM_{PEI}) for forming the polyplexes by electrostatic interactions between the positively charged amine groups of PEI and polyphosphate groups of pDNA, at pH 7.4. The lower MCM_{PEI} value for BPEI (0.8 ng/mL x 10^2) (Figure 1(a)) compared with that in LPEI (1.6 ng/mL x 10^2) (Figure 1(b)) indicates that with a smaller amount of BPEI the complexation of the entire pDNA was possible. This behavior could be related to a higher number of primary amine groups available in BPEI to interact with the pDNA, in addition, due to different three-dimensional (3D) configurations under the HEPES-buffered saline conditions of this study. On the contrary, the polyplexes formation was more efficient when BPEI was used (~100% of complexed pDNA, in contrast with 85% with LPEI (Figure 1(c), 1(d)). Additionally for LPEI/pDNA poly-plexes, at N/P ≥10, the amount of free pDNA remained constant, suggesting that all pDNA had been complexed with the polymer, and that there was polymer in excess in the samples, which could later favor transfection.[25,26]

Moreover, it is possible that PicoGreen dye could reach some pDNA in the LPEI/pDNA polyplexes due to their less compact structure (compared with BPEI/pDNA), resulting from loops and expanded tails of this linear polymer, as discussed in a similar system.[27]

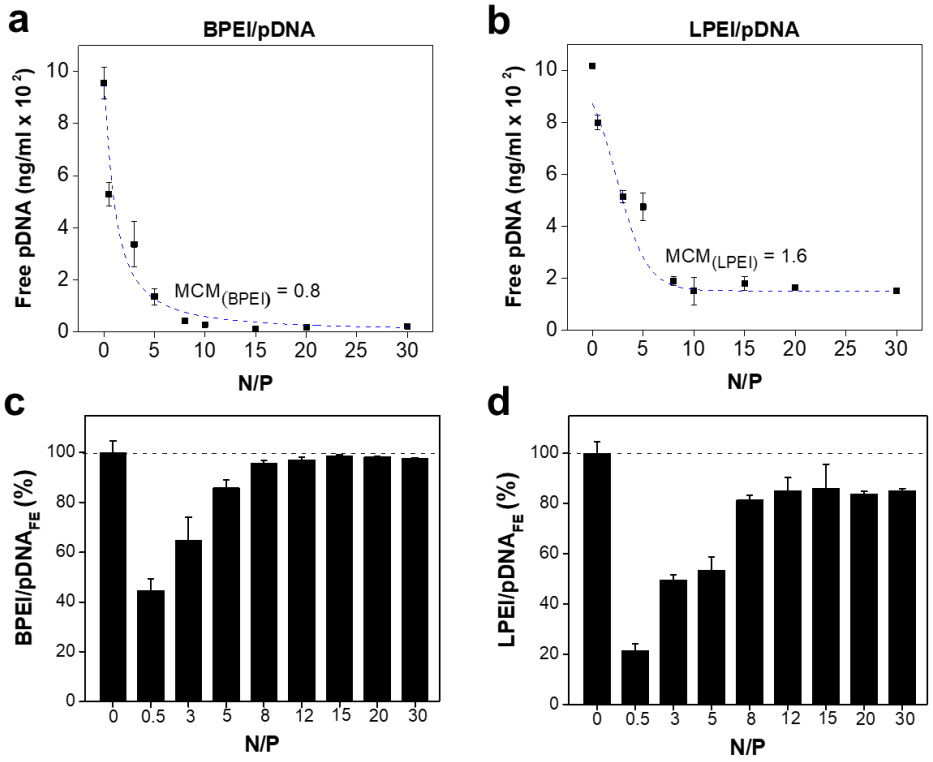

Figure 1. Free pDNA and polyplexes formation efficiency (PEI=pDNA$_{FE}$) in function of N/P ratios for (a,c) BPEI/pDNA and (b, d) LPEI/pDNA samples evaluated from PicoGreen fluorescence assay (ex 480 nm, em 520 nm). Polyplexes were exposed to 100 µl of PicoGreen reagent, and the free pDNA fluorescence was converted to the corresponding DNA concentration according to Equation 1. BPEI, branched polyethylenimine; LPEI, linear polyethylenimine; pDNA, plasmid DNA.

Zeta potential and size of PEI/pDNA polyplexes

The changes of zeta-potential and size for the PEI/pDNA samples were followed in function of the N/P ratios (Figure 2). At N/P ratios < 2.5, the polyplexes had a net negative charge, independent of the polymer type (LPEI or BPEI), possibly for an excess of pDNA in the system. At N/P ratios >5, which were above the MCM$_{PEI}$, the net surface charge is positive. This corroborates the charge screening involved in the PEI/pDNA polyplex formation.

The BPEI/pDNA and LPEI/pDNA samples with N/P ≥15 exhibit zeta potential >30mV, which was correlated with higher colloidal stability due to interparticles electrostatic repulsions.[28,29] This led to nonagglomerated nanoparticles with sizes ranging from 200 to 400nm, as measured by DLS when N/P ratio was 19 (Figure 2(b)). The smaller size of BPEI/ pDNA polyplex nanoparticles could be associated with a higher probability of amine–phosphate group interactions, and therefore, more compact 3D structure formed with this branched polymer, as discussed above.

Considering that a positive surface charge of the polyplexes is necessary to binding to anionic cell surfaces and that small particles are necessary to allow for endocytosis within them, the above results suggest that LPEI/pDNA and BPEI/pDNA polyplexes with N/P ≥ 15 ratios could be a good alternative to carry out the transfection of the epidermal cells.

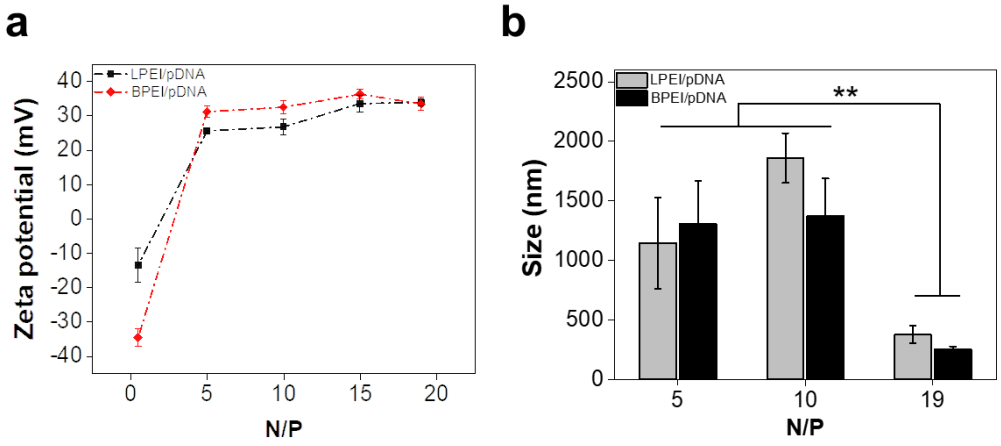

Figure 2. Zeta potential (a) and size (b) of LPEI/pDNA and BPEI/pDNA polyplexes formed in varying N/P ratios (pH 7.4). Polyplexes were prepared in 20mM HEPES–150mM NaCl solution, and water for dynamic light scattering and zeta potential measurements, respectively, mixed and incubated for 10 min before testing. Each data point represents the mean ± standard deviation (n = 3). One-way ANOVA: **p < 0.01.

Cytotoxicity and biocompatibility

The final success of polyplexes as gene delivery carriers is characterized by maximum transfection efficiency and minimal toxicity. The cytotoxicity of LPEI/pDNA and BPEI/pDNA polyplexes was evaluated in keratinocytes and fibroblasts by MTT assay. Untransfected cells were used as controls.

Survival rate in keratinocytes transfected with LPEI/ pDNA at different N/P ratios did not show statistically significant differences compared with the control (Figure 3(a)). When BPEI/pDNA polyplexes were used at N/P ratios between 5 and 15, the survival rate was >100%, and decreased to 70% at the N/P=19 ratio. In fibroblasts (Figure 3(b)), transfection with LPEI/pDNA did not significantly decrease the survival rate in comparison with untransfected cells, on the contrary, at N/P ratios ≥ 10, greater cytotoxicity was observed when BPEI/pDNA polyplexes were used.

To evaluate the biocompatibility of LPEI/pDNA and BPEI/pDNA polyplexes, the hemolysis test in RBCs was performed. As depicted in Figure 3(c), ~20% of the erythrocytes were hemolyzed when treated with both PEIs alone. In contrast, incubation with the polyplexes at various N/P ratios decreased erythrocyte lysis and only led to ~10% hemolysis. These results suggest that the polyplexes could present better systemic compatibility.

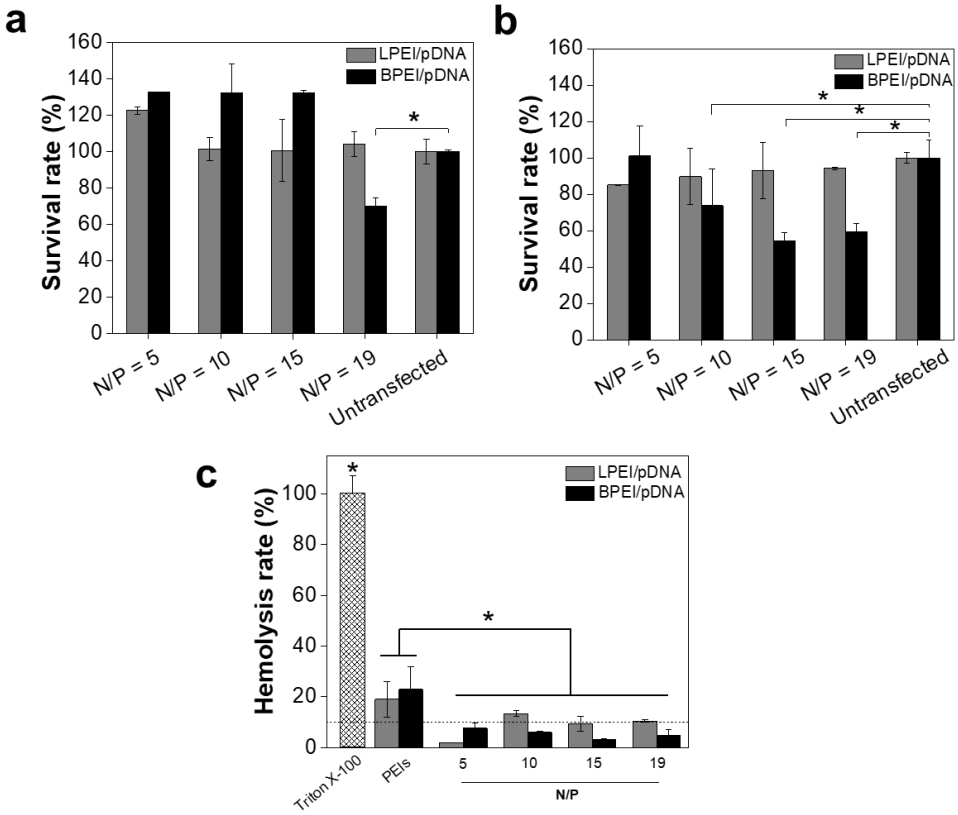

Figure 3. Cytotoxicity in (a) keratinocytes and (b) fibroblasts 24h after transfection with LPEI/pDNA and BPEI/pDNA polyplexes at different N/P ratios versus controls (untransfected cells) by MTT assay. Survival rate was expressed relative to control cells. (c) *In vitro* hemolysis assay of LPEI/pDNA and BPEI/pDNA polyplexes compared with PEIs (LPEI or BPEI alone) and Triton X-100 measured at 460nm. Bar graphs represent the mean ± standard deviation (n = 3). One-way ANOVA: *p<0.05.

Transfection efficiency: LPEI/pDNA versus BPEI/pDNA

In this study, we evaluated nonviral PEI/pDNA polyplex transfection system with N/P=19, comparing two types of polymers (LPEI and BPEI) for effective delivery of the CAMP gene in human keratinocytes and fibroblasts, to overexpress the LL-37 antimicrobial peptide. Flow cytometry analysis and fluorescence micrographs of keratinocytes and fibroblasts transfected with polyplexes (LPEI/pDNA vs. BPEI/pDNA) were used to analyze the polyplex transfection

efficiency (Figure 4). Cells expressing the RFP gene (reporter) were qualitatively observed by fluorescence microscopy (Figure 4(a), and 4(d)). Flow cytometry results confirmed the optical observations (Figure 4(b, c), and 4(e, f)).

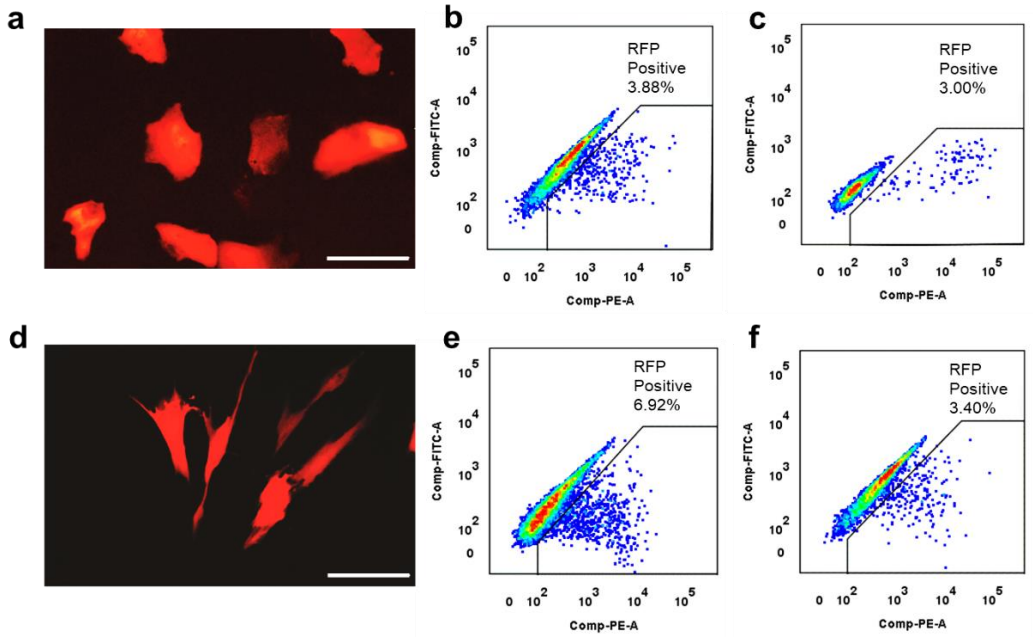

Figure 4. Polyplexes transfection efficiency: LPEI/pDNA versus BPEI/pDNA in (a–c) keratinocytes and (d–f) fibroblasts. (a, d) Detection of positive cells expressing the +RFP by fluorescence microscopy at 24h after transfection (40X). Scale bar = 25 μm. (b, e) FACS analysis of RFP gene expression after transfection with LPEI/pDNA and (c, f) BPEI/pDNA. Polyplexes were generated at N/P=19 and added to the cells. After 6h, the transfection medium was replaced with fresh culture medium. FACS analysis of the cells was performed after 24h. FACS, fluorescence-activated cell sorting; RFP, red fluorescent protein.

Quantitative comparison between the transfection efficiencies and viabilities of keratinocytes and fibroblasts transfected with the selected LPEI/pDNA versus BPEI/pDNA is shown in Figure 5. Results showed significant differences between efficiency medians (percentages of positive cells expressing the reporter gene (+RFP)) in both types of cells. For keratinocytes, a median of transfection efficiency equal to 4.3% and 2.5% was obtained when LPEI/pDNA and BPEI/pDNA were used, respectively. On the contrary, in fibroblasts, an efficiency median of 6.7% and 2.1% was measured with LPEI/pDNA and BPEI/pDNA polyplexes, respectively. Moreover, cell viability after transfection was >70% for keratinocytes and fibroblasts

when BPEI/pDNA polyplexes were used; meanwhile, viabilities were near or > 90% when LPEI/pDNA polyplexes were used.

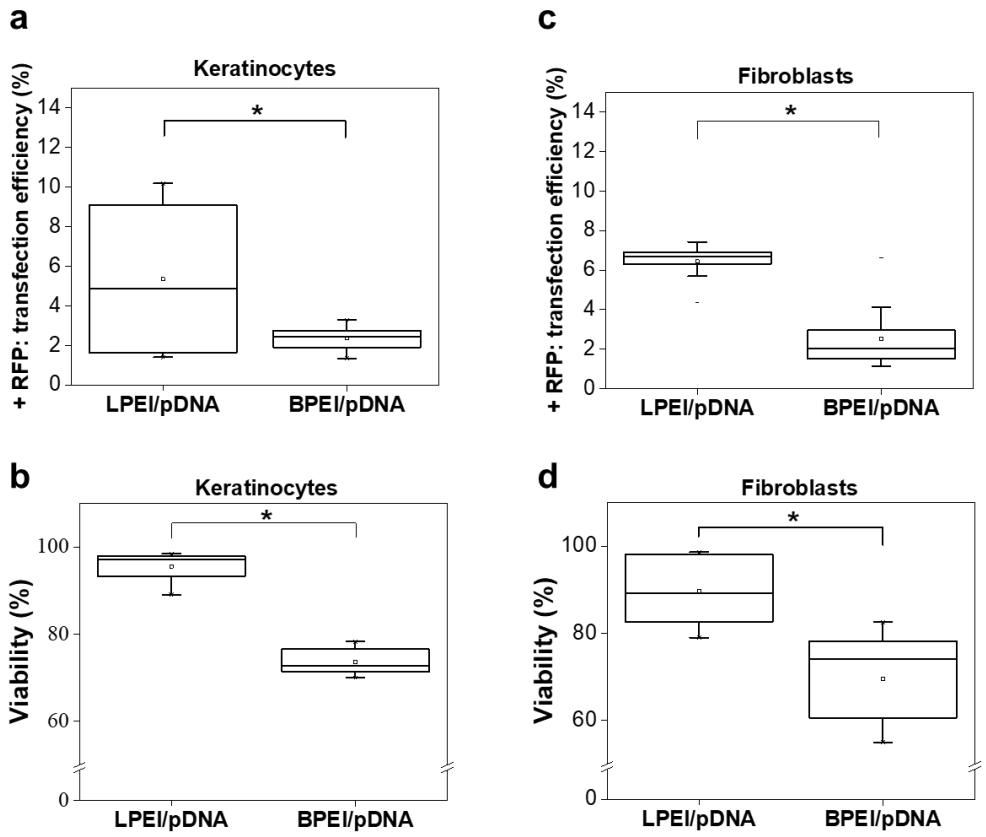

Figure 5. Transfection efficiency and viability comparison between LPEI/pDNA and BPEI/pDNA (a, b) keratinocytes (c, d) fibroblasts. Transfection efficiency was quantified by flow cytometry 24h after transfection. Data from 10.000 events were analyzed and the population of positive cells (+RFP) was selected plotting a diagram ''Comp-FITC versus CompPE-A'' with fibroblasts or keratinocytes that were not transfected (negative control). The cell viability was determined using propidium iodide. Mann–Whitney U test, n=4, *p<0.05.

CAMP gene expression in human keratinocytes and fibroblasts

The results of the CAMP-gene-copy number quantification in human keratinocytes and fibroblasts (Figure 6) show overexpression of CAMP gene for both cell types at 24 and 48h post-transfection (significant differences with respect to the control cells). In keratinocytes, no significant differences were observed between

transfections with LPEI/pDNA and BPEI/ pDNA. In contrast, in fibroblasts, the expression of *CAMP* gene was significantly higher when the transfection was performed using LPEI/pDNA.

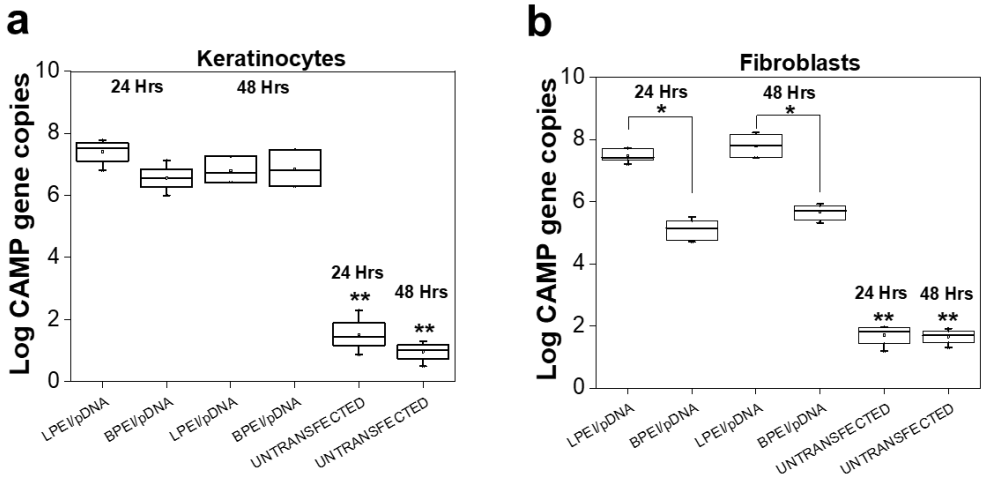

Figure 6. Quantification of CAMP gene expression in (a) keratinocytes and (b) fibroblasts at 24 and 48h post-transfection. The cells were transfected with LPEI/pDNA and BPEI/pDNA, then homogenized with Trizol for the extraction of total RNA. cDNA was synthesized from 2μg of total RNA and CAMP-gene-copy number was quantified by quantitative realtime PCR system using *CAMP*-specific PCR primers. Mann–Whitney U test, n=3, *p<0.05, **p<0.01. PCR, polymerase chain reaction.

Antimicrobial activity in vitro

After confirming the overexpression of the CAMP gene in transfected keratinocytes and fibroblasts with LPEI/pDNA and BPEI/pDNA polyplexes, *in vitro* antimicrobial activity against *S. aureus (ATCC25923)* of culture supernatants collected from keratinocytes and fibroblasts at 24 and 48h after transfection was evaluated. Representative pictures of blood agar plates after 24h of culture are shown in Figure 7. The number of colonies decreased after 1h exposure to the culture supernatants from transfected keratinocytes and fibroblasts with LPEI/pDNA polyplexes (Figure 7(a)), while the supernatants from the cells transfected with BPEI/pDNA polyplexes showed no activity (Figure 7(b)). The CFU/mL count of *S. aureus* showed a decrease when the culture supernatants from both types of cells transfected with LPEI/pDNA polyplexes were used (Figure 7(c)).

Table 1 shows that bacterial growth was decreased by 95.8% and 60% when the microorganism was incubated for 1h with cell supernatants of keratinocytes and fibroblasts transfected with LPEI/pDNA polyplexes, respectively.

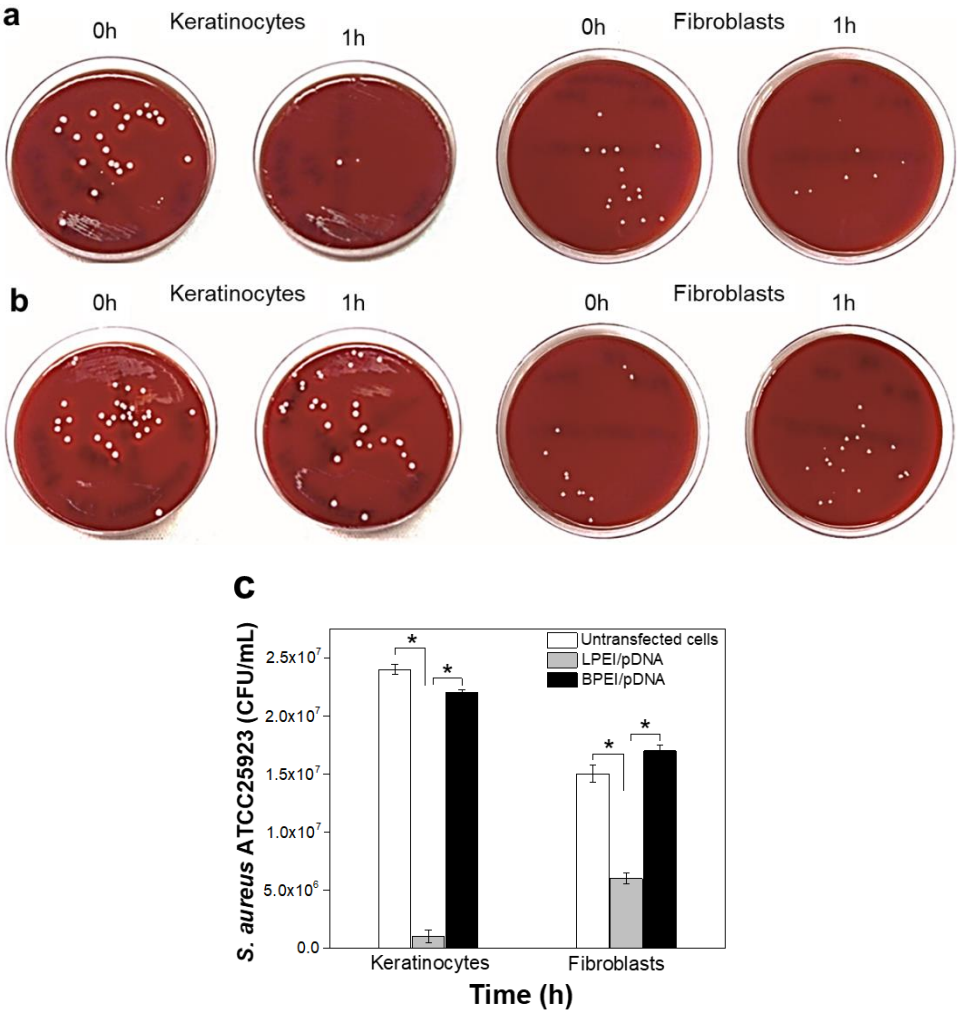

Figure 7. Comparison of antimicrobial activity from cell culture supernatants: LPEI/pDNA versus BPEI/pDNA transfected cells, on the number of viable *Staphylococcus aureus* (CFU). In (a) supernatants from transfected keratinocytes and fibroblasts with LPEI/pDNA decreased the growth of colonies after 1h of contact with the microorganism. In (b) supernatants from both transfected cells with BPEI/pDNA showed no inhibition. (c) Viability of *S. aureus* measured in CFU/mL. The CFU/mL was calculated using the formula: CFU/mL= (no. of

colonies x dilution factor)/volume of culture plate. All graphs depict mean and standard deviation.

DISCUSSION

Skin infections are a significant problem that complicates the definitive closure of wounds. Autologous tissue recovery in these lesions could be ineffective due to the low vascularization in the affected area.[2] Genetically modified dermoepidermal cells can be used to produce temporary skin substitutes to increase the levels of peptides that promote antimicrobial activity in the wound area.[30] The use of branched and linear forms of PEI has been described in various studies to produce polyplexes for gene transfer in primary cells.[13,31,32] However, it is well known that results in transfection depend to a large extent on the type of cells and so far, the behavior of this system in the transfection of skin human cells (fibroblasts and keratinocytes) has not been yet described. In this study LPEI polyplexes were compared with BPEI polyplexes to increase the expression of the *CAMP* gene in primary skin fibroblasts and keratinocytes.

It was reported that higher polymer/DNA ratios can produce compact particles with small sizes and a strong positive net charge, which also facilitate the entry of polyplexes into cells and nuclei.[33–35] In contrast, as reported by Xie et al.,[36] when the N/P ratios are low, particles with diameters between 450 and 950 nm are more frequently found. These large particles do not easily enter the cell, and their structure is not stable enough. The results of this study revealed that for both PEIs, N/P ratio=19 formed complexes with the pDNA, which had significantly smaller diameters than those of lowest N/P ratios, which were mainly agglomerates (Figure 2). This tendency toward aggregation when N/P < 15 (Figure 2(b)) could be explained by the low zeta potential values in contrast with ≥30mV, which is desired charge to achieve sufficiently high repulsion forces between the particles and therefore, an electrostatically stabilized suspension.[28,34] Consequently, these particles could be more stable and interact better with negatively charged cell membranes.

Flow cytometry was used to quantify the efficiency of transfection of LPEI/pDNA and BPEI/pDNA polyplexes in human keratinocytes and fibroblasts. The N/P ratio equal to 19 was selected according to the results previously obtained and the suggestion of Hsu and Uludag.[24] In this N/P ratio a full binding between the polymer and pDNA was confirmed (Figure 1), and the formed polyplexes had a

high cationic net charge with sizes ranging from 200 to 400nm (Figure 2). Although representative images of fluorescence microscopy confirmed the presence of RFP positive cells (+RFP) when both PEIs were used in the transfection (Figure 4(a–d)), efficiencies of transfection and viabilities of keratinocytes and fibroblasts seemed to be better when LPEI/pDNA was used.

However, compared with the controls (untransfected cells), overexpression of the *CAMP* gene was observed in both types of cells after 24 and 48h of transfection (Figure 6). In the transfected keratinocytes, no differences in the *CAMP* expression were observed when LPEI/pDNA and BPEI/pDNA were used (Figure 6(a)), whereas in fibroblasts LPEI/pDNA exhibited a 50% enhancement of *CAMP* gene expression (Figure 6(b)). This suggests that even though the expression of RFP as a reporter gene was low, the final delivery of pDNA in the cells could be sufficient to achieve a high number of copies of the *CAMP* gene. In addition, regulatory elements on the plasmid vector, such as the Internal Ribosome Entry Site Sequence (IRES), which was used to obtain a separate and simultaneous expression of the RFP gene and the CAMP gene, can also affect the expression of genes that are downstream of this sequence (RFP gene in this case).[37] Indeed, we demonstrated this behavior in a previous study, in which the expressions of pDNA and a plasmid that did not contain the IRES sequence upstream of the reporter gene were compared.[10] The results showed that when IRES sequence is driving the expression of the reporter, it significantly decreases the expression in keratinocytes and fibroblasts. Nevertheless, this effect on the reporter gene expression could have positive effects by decreasing the cytotoxicity of the transfection. This could explain why the viability values were always >50% in the evaluated cells.

Both polyplexes exhibit a positive net charge, which is an essential requirement to favor the endocytosis and subsequent delivery of genes into host cells.[38,39] However, the differences in transfection efficiencies can also be explained in terms of the formation efficiency and the free/complexed pDNA exchange in each kind of polyplexes. Even the BPEI polymer is able to complex a higher amount of pDNA than LPEI (Figure 1(a)); the release of pDNA is favored from the less compact tridimensional structure of the LPEI/pDNA polyplexes. Although a complete binding of pDNA with the polymer is an important requirement to maintain its integrity against degradation by nucleases, the release of pDNA from the polyplexes is necessary for the transcription in the cell nucleus.[40,41] For that reason, within the

cytoplasm, LPEI could release pDNA more easily than BPEI. In fact, Itaka *et al*.[42] reported that LPEI has a rapid and efficient pDNA dissociation in the cytoplasm, while the BPEI polyplexes remain in the condensed state even after 24h of transfection, which is unfavorable for final transcription. On the contrary, considering that polyplexes of both PEIs are formed at N/P ratios >10 (Figure 1(a), see N/P > 10), where the amount of free pDNA remains constant, it could be speculated that the presence of free PEI chains can be beneficial for overcoming intracellular traffic barriers, due to their interaction with the lipids of the endosomal membrane.[43–45]

An efficient condensation process of pDNA also depends on PEI molecular weights. Therefore, as the molecular weight of PEI increases, the efficiency of transfection could also increase due to better pDNA protection. However, it is known that high molecular weight polymers such as those evaluated in this study can exhibit higher cytotoxicity, which is an important limitation for their clinical use.[46] According to the results, LPEI/pDNA polyplexes did not show cytotoxicity in the evaluated cells, while BPEI/pDNA significantly decreased the survival rate of keratinocytes at N/P ratios >10 (Figure 3). This is consistent with the reported results in other studies in which LPEI was less toxic than BPEI.[32,47–49] These studies support that LPEI/pDNA polyplexes are less compact than BPEI/pDNA ones, so they can dissociate more easily inside the cell. In fact, LPEI proved to have less affinity for the pDNA, which was confirmed by a higher MCM_{PEI} value compared with its branched counterpart (Figure 1(a)). This observation is consistent with the described mechanism of LPEI-mediated delivery of nucleic acids into the cells. As demonstrated by Rehman *et al*.,[50] the decomplexation of the LPEI polyplexes very likely occurs in the endosomal compartment since, after transfection, nucleic acids are released and accumulated in the nucleus, while the polymer is still located at the site of release.

Moreover, the hemolysis assay was carried out to evaluate the biocompatibility of polyplexes, in terms of the toxicity against the RBC membrane. The results suggest that polyplexes at the N/P ratios evaluated can reduce the hemolysis rate by ≥ 50% compared with the exposure to the PEIs alone. This could facilitate future clinical applications. On the contrary, since this study follows an *ex vivo* approach, where the cells will be transfected before transplantation, the polyplexes would not represent a risk for the future receptor.

Finally, to estimate the production of the LL-37 antimicrobial peptide in transfected keratinocytes and fibroblasts, the ability of cell culture supernatants collected 24h after transfection to inhibit the growth of the planktonic *S. aureus* (strain ATCC25923) was evaluated. As summarized in Table 1, comparison between supernatants from cells transfected with LPEI/pDNA and BPEI/pDNA polyplexes revealed a greater decrease in the bacterial growth when LPEI/pDNA was used in the transfection. Interestingly, the quantification of the CAMP gene by quantitative real-time polymerase chain reaction showed no differences in keratinocytes, therefore no difference would be expected in the amount of secreted LL 37.

Table 1. Antimicrobial activity of cell culture supernatants against *Staphylococcus aureus* (ATCC25923)

Growth inhibition rates (%) against *Staphylococcus aureus* (ATCC25923)	*Transfected keratinocytes*		*Transfected fibroblasts*	
	LPEI	*BPEI*	*LPEI*	*BPEI*
	95.8	No inhibition	60	No inhibition

BPEI, branched polyethylenimine; LPEI, linear polyethylenimine

In fibroblasts, although CAMP expression was significantly lower with BPEI/pDNA polyplexes, LL 37 secretion and some activity against bacteria were still expected. This behavior indicates that there are additional parameters that drive and limit protein production. For example, post-translational modifications can create barriers for protein expression when the PEI is used to mediate transfection. Indeed, two mechanisms have been described that could be involved in protein synthesis repression when there is damage to the integrity of the endoplasmic reticulum due to transfection, namely the unfolded protein response and overload response. In this scenario, the produced proteins can be degraded, or the cells can be eliminated through apoptosis.[33,51] On the contrary, it is also known that the PEI/pDNA polyplexes that remain complexed after transfection could affect healthy cells since they remain as huge clusters in the outer surface of the intracytoplasmic vesicles. In that sense, it is possible that LPEI polyplexes have a lower impact on the cells as previously mentioned; there is evidence that LPEI-mediated cytosolic delivery of DNA occurs in the endosomal compartments and the polymer remains located there even after the DNA is transported to the nucleus.[50] This can favor not only effective transfection but also degradation of the polymer in the lysosomes. Investigating this

mechanism in the BPEI polyplexes could provide a better understanding of the limitations observed in this study.

CONCLUSION

The polyplex system using a LPEI with PS100080-*CAMP* provides a promising strategy for the genetic modification of human fibroblasts and keratinocytes. In the future, these modified cells could lead to more functional skin grafts increasing the levels of antimicrobial peptides as an efficient way to prevent infections in skin wounds.

ACKNOWLEDGMENTS

This work was developed in the facilities of the IPS Universitaria, University of Antioquia, Medellin, Colombia. The authors gratefully acknowledge support from The Molecular Biology and Computational Unit (PECET—Program for the Study and Control of Tropical Diseases) and Dr. Yoshita Bhide at Medical Microbiology and Infection Prevention, University Medical Center Groningen for help with the hemocompatibility assays.

DISCLOSURE STATEMENT

No competing financial interests exist.

FUNDING INFORMATION

This work was supported by COLCIENCIAS, National Doctoral program (conv 727-2015).

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Chapter 4

Nonviral Expression of LL-37 in a Human Skin Equivalent to Prevent Infection in Skin Wounds

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Human Gene Therapy 2021; doi: 10.1089/hum.2021.034.

ABSTRACT

Inefficient autologous tissue recovery in skin wounds increases the susceptibility of patients to infections caused by multidrug resistant microorganisms, resulting in a high mortality rate. Genetic modification of skin cells has become an important field of study because it could lead to the construction of more functional skin grafts, through the overexpression of antimicrobial peptides that would prevent early contamination and infection with bacteria. In this study, we produce and evaluate Human Skin Equivalents (HSEs) containing transfected human primary fibroblasts and keratinocytes by polyplexes to express the antimicrobial peptide LL-37. The effect of LL-37 on the metabolic activity of normal HSEs was evaluated before the construction of the transfected HSEs, and the antimicrobial efficacy against *Pseudomonas aeruginosa* and *Staphylococcus aureus* was evaluated. Subsequently, the levels of LL-37 in the culture supernatants of transfected HSEs, as well as the local expression were determined. It was found that LL-37 treatment significantly promoted the cellular proliferation of HSEs. Furthermore, HSEs that express elevated levels of LL-37 were shown to possess histological characteristics close to the normal skin and display enhanced antimicrobial activity against *S. aureus in vitro*. These findings demonstrate that HSEs expressing LL-37 through non-viral modification of skin cells is a promising approach for the prevention of bacterial colonization in wounds.

INTRODUCTION

Healthy and intact skin acts as a barrier that prevents the entrance of microorganisms from the environment.[1] Wounds caused by mechanical trauma, surgical interventions, chronic wounds, burns, or the natural aging compromises the integrity of this barrier and favors bacterial contamination, which can complicate wound closure and patient recovery, increasing morbidity and mortality, as well as the socioeconomic impact worldwide.[2,3]

Opportunistic pathogens such as *Pseudomonas aeruginosa* and *Staphylococcus aureus* are able to infect open wounds and produce biofilms which, once established, protect the bacteria against the effects of commonly used antibiotics. Consequently, chronic wounds infected with populations of resistant bacteria can develop that compromise the lives of patients.[4,5,6] Multiple dressing formats have been commonly implemented to inhibit microbial proliferation or treat local infections. Broad-spectrum antimicrobial agents can be combined with dressing materials such as foams, hydrogels, alginates, collagens, or films.[7,8] However, because of increasing antimicrobial resistance, these agents cannot be used for extended periods of time. Autologous skin grafts are also used for the treatment of these lesions, nevertheless, they are limited by the availability of healthy donor tissue in cases of large skin loss.[9] Therefore, the development of innovative strategies to prevent and combat infected cutaneous wounds is still needed.[10,11]

The physical barrier provided by the skin is augmented by the innate immune system and the expression of the host defense peptides (HDPs), which act as a chemical barrier to reinforce the protection of the tissue. However, the wound environments are often depleted of HDPs, which can decrease the early response against microorganisms. HDPs are produced mainly by keratinocytes of the epidermis. In mammalian species, the two major classes of HDPs are the defensins and cathelicidins. The linear peptide LL-37 is the only member of the cathelicidins family identified in humans.[12,13] The expression of LL-37 is regulated by the transcription of the *CAMP* (cathelicidin antimicrobial peptide) gene, which translates to an 18 kDa proprotein known as hCAP18 (human cationic antimicrobial protein), that releases the active C-terminus 37 amino acid peptide after the post-translational processing. In addition to its antimicrobial activity against a variety of bacteria,[14,15] LL-37 is involved in the chemotaxis of immune and endothelial cells, as well as keratinocyte proliferation during the wound healing processes.[16] For these

reasons, LL-37 has been a promising tool for wound treatment development as an alternative to conventional antibiotics. However, the therapeutic application of synthetic LL-37 in clinical practice has been hindered by the high cost of peptide synthesis and biological disadvantages, such as their susceptibility to proteolysis in the wound bed and their poor activity under physiological conditions.[17]

Currently, human skin equivalents (HSEs) offer another alternative for the treatment of skin wounds. HSEs are three-dimensional models based on biopolymers, in which different cell populations can be incorporated to favor the covering of the lesion and provide a bed for new cells to regenerate the lost tissue. Biopolymers such as fibrin have been used successfully for the construction of HSE, as it can stimulate the production of extracellular matrix (ECM) by fibroblasts, providing an environment that promotes tissue regeneration.[18,19] Moreover, gene therapy has allowed to genetically modify the skin cells to increase the production of antimicrobial peptides such as the LL-37.[20] These modified cells can be incorporated in the HSEs to function as "bioreactors" *in vivo*. The gene transfer to epidermal cells has been commonly performed through retroviral methods, which despite its high efficiency, have disadvantages such as immunogenicity, oncogenicity and development of mutagenic processes.[21,22]

Consequently, the use of synthetic non-viral vectors has generated a great interest in protocols for clinical use in gene therapy. In particular, polyethylenimine (PEI) polyplexes have been widely used for non-viral transfection in mammalian cells due to their ability to protect nucleic acids against premature degradation, and thus facilitate their delivery through the plasma membranes.[23,24] This system, together with the use of scaffolds such as HSEs, allows maintaining the *in vitro* expression of the protein of interest and reducing the cytotoxicity that some of these systems present against the target cells. In addition, the development of HSEs with modified autologous cells could provide new and improved properties by targeting the excretion of those proteins of interest specifically to the site of action, preventing any systemic side effects such as immune recognition and rejection.[25]

In this report, we first investigate the antimicrobial efficacy of the synthetic LL-37 peptide and its effects on human primary fibroblasts and keratinocytes. Then, we demonstrate that HSEs generated from skin cells genetically modified by linear polyethyleneimine (LPEI) polyplexes express elevated levels of the LL-37 and possess enhanced antimicrobial activity *in vitro*. This approach could support the

coverage of cutaneous wounds and prevent bacterial growth, which could keep the wound bed in more sterile conditions favoring the closure and recovery of the tissue.

MATERIALS AND METHODS

Materials

Synthetic LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES) was purchased from PolyPeptide group (Malmö, Sweden) at ≥ 90% purity, confirmed by high-performance liquid chromatography (HPLC). Linear PEI (LPEI 25 kDa) was purchased from Polysciences and the recombinant plasmid containing the CAMP sequence coding to the hCap18/LL-37 expression was purified from existing *Escherichia coli* stocks, using a plasmid maxi kit (Qiagen EndoFree®). The plasmid (hereafter called pDNA) allows the expression of both the reporter gene (Red Fluorescent Protein [RFP]) and the gene of interest (*CAMP*). Human Dermal Fibroblasts (NHDF) and Epidermal Keratinocytes (NHEK) tested and certified as mycoplasma-free and virus-free (HIV-1, hepatitis B, and hepatitis C) were purchased from Lonza, The Netherlands. The reagents for cell culture such as Dulbecco's modified Eagle's medium (DMEM) High glucose, the nutrient mixture F-12 Ham (Ham's F-12), and fetal bovine serum (FBS) were purchased from Gibco, Thermo Fisher Scientific, USA.

Bacterial strains and culture conditions

S. aureus ATCC 25923 and *P. aeruginosa* PAO1 were grown from stock solutions (7% dimethylsulfoxide [DMSO], at −80°C) on blood agar plates at 37°C for 24 h. Bacteria were cultured to late-stationary phase in tryptic soy broth (TSB, OXOID, Basingstoke, UK) as described below. Single colonies were inoculated into 10 mL TSB broth and incubated overnight at 37°C. Then, this pre-culture was inoculated into 200 mL TSB broth in the absence or presence of 50 mM sodium bicarbonate ($NaHCO_3$, pH 7,4) in order to evaluate the impact of bicarbonate on the antimicrobial potency of the LL-37 peptide. Bacterial cultures were grown for 24 h at 37°C with continuous rotation (200 rpm) and harvested by centrifugation for 5 min at 5000g. After removal of the broth, bacterial pellets were washed twice in phosphate buffered saline (PBS, 5 mM K_2HPO_4, 5 mM KH_2PO_4, 150 mM NaCl, pH 7.4), and sonicated for three times 10 s (Vibra cell model 375, Sonics and Material Inc). The final concentrations were adjusted to 1×10^5 bacteria/mL using a Bürker-Türk counting chamber.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays

The minimum inhibitory concentrations (MIC) of the synthetic LL-37 peptide were determined using the broth microdilution method. Briefly, two-fold dilution series of the peptide starting from 256 μM to 0.25 μM were prepared in 96-well plates in PBS. In each well, 100 μL of a bacterial suspension containing $1x10^5$ bacteria/mL prepared in absence or presence of 50 mM sodium bicarbonate (pH 7.4) were added. The 96-well plates were incubated at 37°C for 24 h and the MIC was determined as the lowest peptide concentration at which bacterial growth was not visually observed. Subsequently, the minimum bactericidal concentrations (MBC) were determined from the broth dilution of MIC assays (starting from the MIC values with no visual growth) by inoculating 10 μL on TSB agar plates. The agar plates were incubated for 24 h at 37°C. The lowest concentrations of LL-37 at which no colonies were seen on the plates were defined as the MBC.

Also, the absorbances from the wells were recorded after 24 h of incubation using a spectrophotometer (Shimadzu, Japan) at 600 nm to compare bacterial growth in the absence and presence of sodium bicarbonate with the respective negative controls, which consist of the bacterial suspension without peptide exposition or 0 μM LL-37. Three independent experiments with separately cultured bacteria were performed, each with three replicates.

Cell cultures and generation of human skin equivalents (HSEs)

Fibroblasts were cultured in DMEM high glucose supplemented with 10% (v/v) FBS and 1% (v/v) penicillin-streptomycin. Keratinocytes were cultured on a mitomycin C-treated 3T3 feeder layer and DMEM: Ham's F-12 (3:1) supplemented with 10 v/v % FBS and 10 ng/mL epidermal growth factor (EGF, Austral Biologicals, USA). Only passages between 1 and 5 were used for all experiments.

The generation of the HSEs was performed according to the protocol established by the Group of Tissue Engineering and Cellular Therapy, University of Antioquia, Colombia albeit with some modifications.[26] In brief (Figure 1), a mixture of pooled human plasma (blood derived from donors), 150 mM NaCl, 5 μL of 5 mg/mL tranexamic acid (CAS Number 1197-18-8, Sigma), and a fibroblast suspension ($2x10^5$ cells/mL) was prepared. Then, 1% $CaCl_2$ (Sigma) was added to promote fibrin polymerization, and after gently mixing the solution, it was poured

into cell culture inserts (Transwell®, Corning 3460) with 0.4-μm porous membranes, and incubated at 37°C, 5% CO_2 for 30 min. Subsequently, each fibrin gel was covered with DMEM, high glucose supplemented with 10% FBS, and incubated overnight at 37°C. Afterward, a keratinocyte suspension ($2x10^5$ cells/ 1.2 cm^2) was seeded on top of each gel in the transwell, to obtain keratinocytes vs fibroblasts ratio of 3:1. The cultures were kept immersed in the cell culture medium for 7 days, refreshing the medium every 3 days. Next, the medium from the "epidermal" zone in the transwell was removed and the keratinocytes were exposed to the air-liquid interface for 14 days at 37°C, and 5% CO_2 to stimulate the stratification.

Generation of HSEs modified by LPEI/pDNA polyplexes

Figure 1 shows the culture protocol overview of HSEs with transfected skin cells. Briefly, keratinocytes and fibroblasts were seeded separately 24 h before transfection into 24-well plates (5×10^3 cells/cm^2) and incubated at 37°C in 5% CO_2. The transfection procedure was carried out using a protocol previously published.[27,28] LPEI/pDNA polyplexes were initially prepared in 4-(2-hydroxyethyl) piperazine-1-ethane-sulfonic acid (HEPES)-buffered saline (150 mM NaCl, 20 mM HEPES, pH 7.4) with a N/P ratio of 19 (molar ratio of nitrogen in LPEI [6.3 μg] to phosphorus in pDNA [2.5 μg]). Next 50 μL of this polyplexes solution was mixed with 0.5 mL DMEM: Ham's F-12 (3:1) low in serum (1% FBS) without antibiotics. Subsequently, the plates were centrifuged at 210 g for 5 min and the cells were incubated with the polyplexes for 6 h at 37°C, and 5% CO_2. Finally, the medium was replaced with fresh medium to remove the remaining polyplexes, and the cells were incubated for an additional 24 h.

The cells were visualized using fluorescence microscopy (Leica DMR Leica, Wetzlar, Germany) to confirm the expression of the Red Fluorescent Protein (RFP) reporter gene and the HSEs were constructed as previously described with a final volume of 0.5 mL.

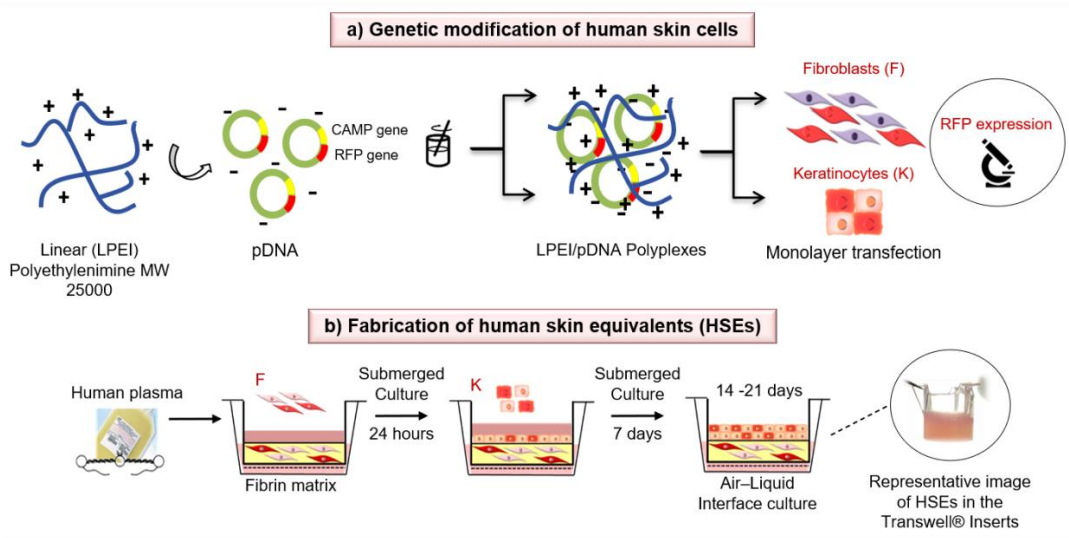

Figure 1. Schematic representation of the process for fabrication of HSEs with transfected cells. In (a) LPEI/pDNA polyplexes with N/P ratio = 19 (6.3 μg LPEI per 2.5 μg pDNA) were prepared and added to fibroblast and keratinocyte monolayers. In (b) the dermal matrix was prepared by encapsulating primary human fibroblasts within a fibrin-based 3D matrix. After 24 h, the keratinocytes were seeded on top of the dermal matrix populated with fibroblasts and cultured for 7 days under immersion conditions. At day 7 of culture, the HSEs were exposed to the air-liquid interface until day 21. Negative controls were generated under the same protocol using non-transfected cells. 3D, three-dimensional; HSEs, human skin equivalents; LPEI, linear polyethyleneimine.

Effects of LL-37 on human skin equivalents

To examine the effect of LL-37 peptide, the metabolic activity of HSEs at different stages of the cell culture were determined. Samples of HSEs were prepared as described above and cultured in cell growth medium [DMEM: HAM F12 (3:1) supplemented with 10% FBS] at 37°C and 5% CO_2 atmosphere. The growth medium was mixed with decreasing concentrations of LL-37 (two-fold dilutions from 256 μM to 2 μM) and then added to the HSEs for 1, 7, and 14 days. The medium was replaced with fresh medium supplemented with LL-37 every 3 days. Subsequently, a solution of 500 μL of XTT [(2,3-bis (2-methoxy-4-nitro-5-sulfophenyl) -2H-tetrazolium-5-carboxanilide salt), A8088 AppliChem, The Netherlands] was added to the growth medium present on top of each sample. After 6 h of incubation at 37°C, 100

μL of the mixture was taken from each sample and absorbances A460nm and A690nm were measured using a spectrophotometer (Shimadzu, Japan). The metabolic activity of the LL-37 treated HSEs was calculated relative to the negative control samples (HSEs exposed to growth medium in the absence of LL-37) according to Equation 1.

$$\text{Metabolic activity (\%)} = \frac{A_{HSEs}\,460nm - A_{HSEs}\,690nm}{A_{Negative\ controls}\,460nm - A_{Negative\ controls}\,690nm} \times 100 \qquad [1]$$

The absorbance of all samples was corrected by subtracting the culture medium background from samples that do not contain cells and were treated identically as the HSEs exposed to LL-37 or negative controls.

To study the effect of LL-37 on cell proliferation of HSEs, a group of samples exposed to various concentrations of the peptide were fixed with a 3.7% paraformaldehyde (Sigma) solution for 1 h and subsequently washed with PBS three times. Then, the samples were permeated using a 0.5% Triton X-100 (Sigma) solution for 3 min and blocked for nonspecific binding with 5% bovine serum albumin (BSA, Sigma) in PBS (PBSA) for 30 min. Afterward, the samples were stained using 4′, 6-diamidino-2-phenylindole (DAPI) (Sigma, D9564, ratio: 1:500), and tetramethylrhodamine isothiocyanate (TRITC)-phalloidin for visualization of the nucleus and cytoskeleton of the cells, respectively. Cells were visualized with a fluorescence microscope (Leica DMR, Wetzlar, Germany) after 14 days in culture and the number of cells was reported as the number of nuclei in 0.5 mL of gel.

Enzyme-linked immunosorbent assay (ELISA)

To test whether microbial stimulation increases the expression of the LL-37 peptide, culture supernatants were collected from monolayers of fibroblasts and keratinocytes transfected and non-transfected, cultured for 7 days. For the stimulation, a bacterial culture suspension was prepared by inoculating $1x10^5$ bacteria/mL *S. aureus* ATCC 25923 in TSB overnight, then, bacteria were harvested by centrifugation for 5 min at 5000g and the supernatant was carefully removed, and filter sterilized using a standard PTFE filter (Millipore, PTFE, 0.22 μm). The bacterial spent medium was mixed in DMEM–TSB (9:1) and poured on top of the cell monolayers. Then, the cultures were incubated for 24 h, at 37°C and 5% CO_2.

In addition, cell culture supernatants from transfected and nontransfected HSEs, cultured for 14 and 21 days, were collected.

Concentrations of LL-37 in the culture supernatants from both cell monolayers and HSEs were quantified with a commercially available hCAP-18/LL-37 ELISA kit (Hycult Biotech, The Netherlands) using a plate reader (Shimadzu, Japan) according to the manufacturer's protocol. Cell supernatants from three independent experiments were analyzed.

Histology and immunohistochemistry (IHC)

HSEs and transfected HSEs cultured for 21 days were fixed in 3.7% (v/v) paraformaldehyde. The fixed tissues were paraffin embedded or frozen in Tissue-Tek® O.C.T. Compound (Sakura Finetek Europe B.V., Alphen aan den Rijn, The Netherlands) for histological and immunofluorescent analysis, respectively. Paraffin-embedded blocks were sectioned into 5-μm thickness samples and stained with hematoxylin & eosin (H & E). Frozen HSEs were cryosectioned to 7 μm thick slices in a Slee MNT cryotome, fixed in acetone at -20°C and air-dried. Next, the sections were incubated with a blocking solution (2% BSA in PBS) for 30 min and then incubated for 1 h at 37°C with rabbit polyclonal antibodies against LL-37 (1:150) (Innovagen, Sweden). Afterward, samples were carefully washed in PBS and exposed to polyclonal swine anti-rabbit immunoglobulins HRP (1:100) (Dako) (1% BSA in PBS) for 45 min at room temperature. Sections were counterstained with a Hematoxylin and Eosin (H&E) solution, and examined using an inverted microscope (Leica DMR, Wetzlar,Germany). Sections incubated only with the secondary antibody were included as negative controls (Figure S1).

Antimicrobial activity of HSEs

To test the antimicrobial activity of the transfected and non-transfected HSEs, an *in vitro* bacterial inoculation and growth assay was performed. Under sterile conditions, HSEs with a final volume of 0.5 mL cultured for 21 days were inoculated with 1 mL of a solution of $1x10^5$ bacteria/mL (*S. aureus* ATCC 25923) in DMEM supplemented with 10% TSB medium. Only sterile medium was used as a negative sterility control. The co-cultures were incubated for 24 h at 37°C and 5% CO_2. Subsequently, the culture supernatants and the HSEs were collected and separately processed to determine the number of CFU/mL.

After removing the HSEs from the wells, they were weighed and transferred to sterile tubes (Falcon™). The HSEs were put in 0.5 mL PBS and homogenized by sonication (Transonic TP 690, ELMA, Germany, 160 W, 35 kHz) for 30 s at room temperature to achieve the dispersion of the bacteria. Then, 10 μL from the resultant solution was plated on TSB agar plates and incubated for 24 h at 37°C.

RESULTS

Synthetic LL-37 possesses in vitro antibacterial activity against planktonic S. aureus and P. aeruginosa

Table 1 shows MIC and MBC values determined for the synthetic LL-37 against *S. aureus* ATCC 25923 and *P. aeruginosa* PAO1 planktonic bacterial strains in PBS with and without sodium bicarbonate (50 mM, pH 7.4). Sodium bicarbonate is the dominant buffer in the human body and it has been shown to enhance the *in vitro* activity of various mediators of host defenses, including cathelicidins.[29] For *S. aureus*, the MIC of LL-37 was determined to be 32 μM in the presence of bicarbonate, however, the displayed MBC was higher than the highest concentration evaluated (>256 μM). Interestingly, the MIC and MBC values for *P. aeruginosa* did not differ regardless of the addition of sodium bicarbonate. These results confirm those reported in previous studies in which sodium bicarbonate was shown to have an effect on the susceptibility of *S. aureus* due to changes in the bacterial membrane that enhance the permeabilization by cationic compounds as the LL-37.[30]

Table 1. The minimum inhibitory concentration and minimum bactericidal concentration of LL-37 against planktonic strains of *Staphylococcus aureus* ATCC 25923 and *Pseudomonas aeruginosa* PAO1.

Microorganism	*Staphylococcus aureus ATCC 25923*		*Pseudomonas aeruginosa PAO1*	
	MIC (μM)	*MBC (μM)*	*MIC (μM)*	*MBC (μM)*
(+) $NaHCO_3$	32	>256	64	64
(-) $NaHCO_3$	>256	>256	64	64

MBC, minimum bactericidal concentration; MIC, minimum inhibitory concentration.

In addition, these findings also coincided with the dose response curves (Figure S2) wherein the bacterial growth was evaluated related to the optical density values at OD_{600}. The absorbances from *S. aureus* decreased significantly in the presence of NaHCO3 from 32 μM, whereas for *P. aeruginosa*, LL-37 demonstrated a stimulated growth at low LL-37 concentrations and showed to be completely bactericidal from 64 μM even in the absence of $NaHCO_3$.

Increasing concentrations of LL-37 enhance cell proliferation in HSEs

Fibroblasts and keratinocytes are the major cell types that respond to the inflammatory phase during the cutaneous regeneration process. The response of these cells to the inflammatory signals activates cell proliferation in the wound bed, which is a critical factor to facilitate wound closure. To evaluate the ability of the synthetic LL-37 peptide to induce cell proliferation in HSEs, the HSEs were constructed and cultured in the presence of increasing concentrations of the peptide for 1, 7, and 14 days.

Figure 2(a) shows that compared to the control (0 μM LL-37), the metabolic activity of the HSEs increased when exposed to concentrations up to 64 μM of LL-37 after 7 and 14 days in culture. No differences were observed at higher concentrations (128 μM and 256 μM), however, the values remained close to or greater than 100%. DAPI nuclear staining was used to quantify cell number (Figure 2(b)) in HSEs exposed to the MIC concentrations of LL-37 against planktonic *S. aureus* ATCC 25923 and *P. aeruginosa* PAO1 (32 μM and 64 μM, respectively). After 14 days in culture, the number of cells displayed significant difference among controls and treated HSEs. These results were confirmed by the fluorescence images (Figure 2(c)), which demonstrated an increased number of nuclei and a greater amount of actin filaments. Taken together, these results suggest that cells cultured in HSEs proliferated better in the presence of LL-37.

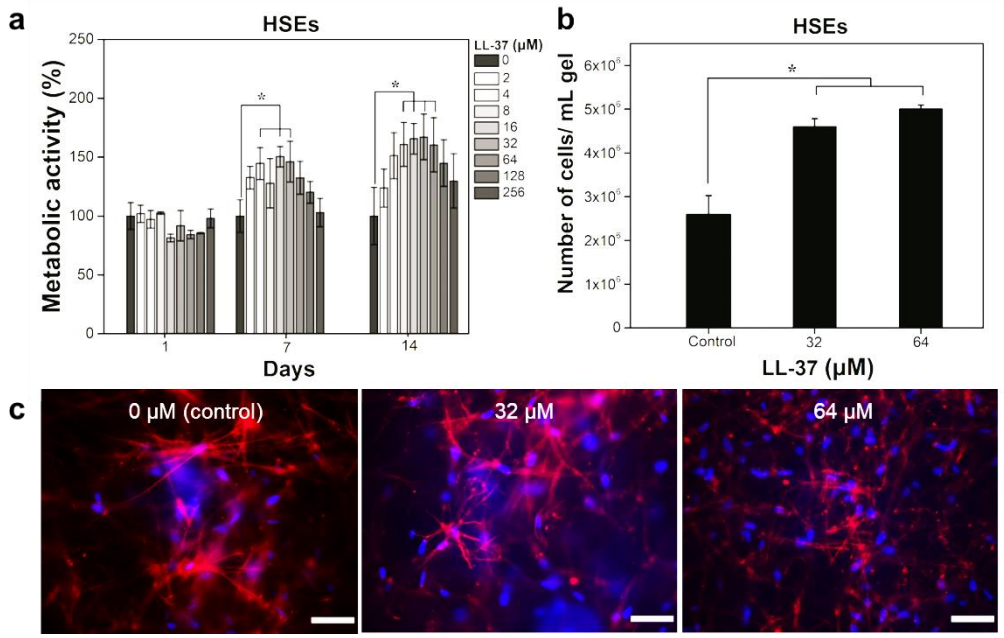

Figure 2. Effect of LL-37 on cell proliferation and metabolic activity in HSEs. (a) HSEs were treated with different concentrations of LL-37 for 1, 7 and 14 days. After 7 days, the metabolic activity assessed by XTT assay increases in a dose-dependent manner. And 0 μM LL-37 was set to 100%. (b) The number of cells in the HSEs after exposure to different concentrations of LL-37 determined by counting the nuclei stained by DAPI using fluorescence microscopy. (c) Representative images of stained cells by phalloidin/DAPI after 14 days in culture at 0 μM, 32 μM, and 64 μM of LL-37 (red = phalloidin, blue = DAPI). Scale bar = 50 μm. Data represent the means ± standard deviation (n = 3). One-way ANOVA followed by Tukey's HSD test: *, $p < 0.05$. ANOVA, analysis of variance; DAPI, 4′,6-diamidino-2-phenylindole; HSD, honestly significant difference.

LPEI/pDNA polyplexes increase LL-37 secretion in skin cells and HSEs in response to bacterial stimulation

The LL-37 peptide levels were first determined by ELISA in cell supernatants from both transfected and non-transfected monolayers of fibroblasts and keratinocytes. In the absence of bacterial stimulation, significant differences in the expression of LL-37 between transfected and non-transfected cells were only observed for keratinocytes (Figure 3(a) and (b)), possibly because LL-37 is endogenously synthesized by keratinocytes and its secretion was increased due to the transfection.

The effect of stimulation with *S. aureus* ATCC 25923 metabolites (spent medium) for 24 h on the secretion of LL-37 in transfected and non-transfected fibroblasts and keratinocytes showed that the secretion of LL-37 by transfected cells was significantly increased compared with non-transfected cells (Figure 3(a) and (b)). Figures 3(c) and (d) show that transfected and nontransfected fibroblasts and keratinocytes morphology was not altered when cultured under bacterial stimulation. This indicates that the increase in LL-37 levels is due to the metabolic response to stress by the cells in contact with bacterial metabolites, and not to the modification of the culture conditions or proliferation. To increase the level of LL-37 peptide in HSEs, monolayer cultures of fibroblasts and keratinocytes were genetically modified with LPEI/pDNA polyplexes, after which the transfected cells were included in the HSE protocol.

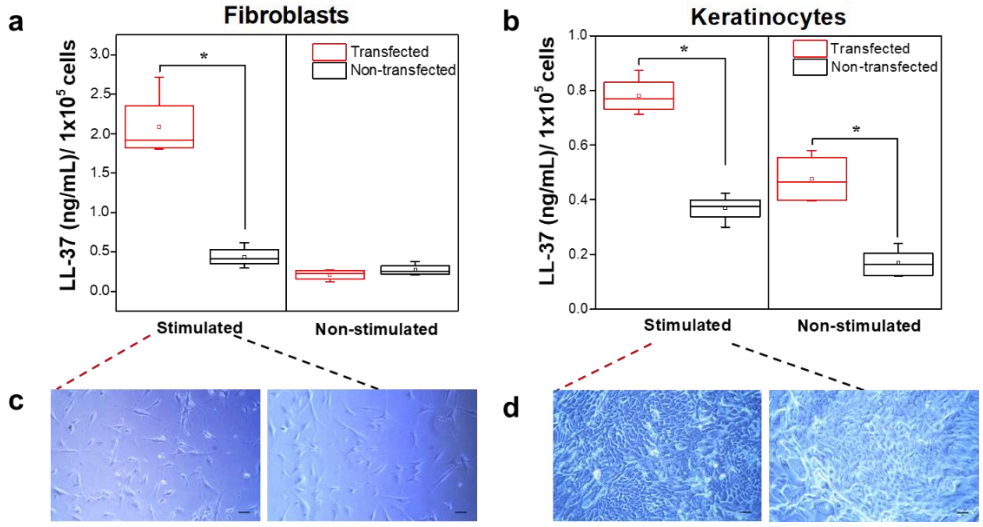

Figure 3. LL-37 peptide expression in cell culture supernatants of genetically modified (a) fibroblasts and (b) keratinocytes quantified by ELISA. After transfection, the cells were cultured in DMEM-TSB (9:1) with and without *Staphylococcus aureus* ATCC 25923 metabolites (spent medium) for 24 h, at 37°C and 5% CO_2. Data are displayed as box-and-whisker plots showing median (line within box), IQR (box), and minimum to maximum (whiskers), (n = 3). Mann–Whitney U-test. *, $p < 0.05$. (c-d) Representative light microscopy images of transfected cells and nontransfected after stimulation with *S. aureus* metabolites for 24 h. 20x. Scale bars 100 μm. DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; IQR, interquartile range; TSB, tryptic soy broth.

After 14 and 21 days in culture, transfected HSEs were exposed to bacterial metabolites for 24 h and cells supernatants were collected and analyzed by ELISA. Figure 4 shows that metabolites from bacteria were able to induce the secretion of significant levels of the LL-37 peptide in HSEs constructed with transfected cells compared to the nontransfected cells after 14 days in culture. Cell culture supernatants collected at day 21 did not show significant differences between the groups, however, the LL-37 expression in HSEs with transfected cells is higher than those of non-transfected by ~10%.

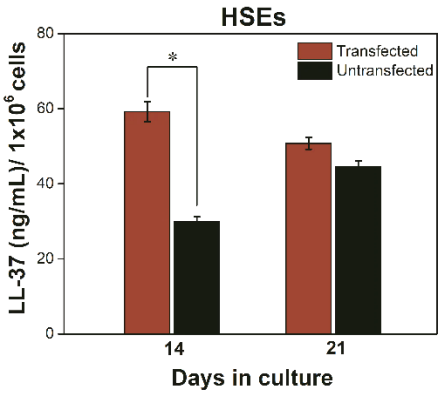

Figure 4. LL-37 peptide excretion by HSEs with transfected cells and controls (non-transfected) stimulated with *S. aureus* ATCC 25923 metabolites detected by ELISA after 14 and 21 days in culture. Note that the cell numbers on the y-axis are combined keratinocytes and fibroblasts. The means ± standard deviation are shown (n = 3, independent experiments). Mann–Whitney U-test. *, $p < 0.05$.

HSEs expressing LL-37 resembles human native skin

After 21 days of culture at the air/liquid interface, the localization of LL-37 in HSEs constructed with transfected and nontransfected cells was explored. Cryosections from both tissues were immunostained using an antibody against the LL-37 peptide (Figure 5). Figure 5(b) shows that the transfected HSEs displayed staining in the basal layer of the epidermis, as well as in some areas of the dermis, while the nontransfected HSEs were stained only in the epidermis (Figure 5(a)), which could be related to the endogenous expression of LL-37 in the keratinocytes from the human skin.[31]

Furthermore, histological evaluation by H&E staining showed that HSEs constructed with transfected cells have a similar architecture to those with non-transfected cells, and both types of HSEs showed characteristics close to the native human skin (Figure 5(c)), with a dermal matrix populated by fibroblasts, and a partially stratified epidermis. These results suggest that there are no alterations in the formation of the dermal and epidermal compartments of the HSEs as a result of the increased LL-37 transgene expression. In addition, the localization of LL-37 in both transfected and nontransfected samples is consistent with the pattern displayed by normal skin tissues, in which LL-37 is expressed mainly in the basal layer.[32]

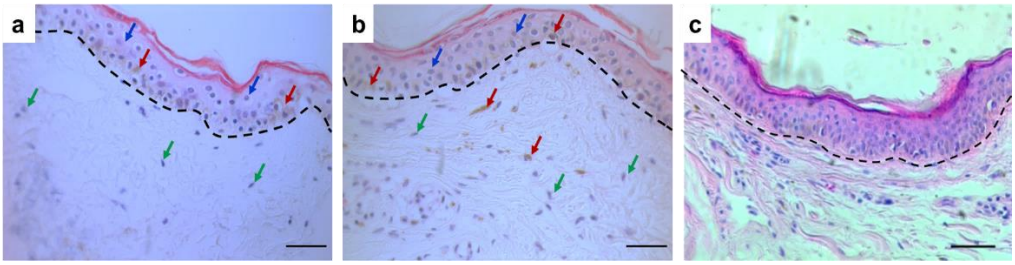

Figure 5. HSEs expressing LL-37 exhibit a similar architecture to native skin and produce LL-37 in both the epidermis and dermis. (a) HSEs created with nontransfected keratinocytes and fibroblasts and (b) HSEs created with transfected keratinocytes and fibroblasts. A separation between dermis and epidermis (dashed line), different layers of keratinocytes (blue arrows), and a dermal layer, which is populated by fibroblasts (green arrows), are shown. HSEs with high expression of LL-37 are indistinguishable from the nontransfected HSEs and both are similar to native skin (c) as depicted in the representative image. Nontransfected HSEs show a low level of LL-37 only in the epidermis, whereas the transfected HSEs produce higher LL-37 expression present both in the epidermis layer and in the dermis (red arrows). Scale bars = 50 μm.

Higher levels of LL-37 in HSEs enhance antimicrobial activity in vitro

To assess the antimicrobial activity *in vitro* of HSEs generated with transfected and non-transfected fibroblasts and keratinocytes, HSEs were cultured for 21 days. Then, the cell culture media was challenged with *S. aureus* ATCC 25923 in growth medium containing $1x10^5$ bacteria/mL. The bacteria were cultured together with the HSEs for 24 h. Figure 6(a) shows that HSEs constructed with transfected cells significantly decreased bacterial growth by approximately one log compared to the HSEs with non-transfected cells. Additionally, the CFU/mL

recovered from the cell culture supernatants from HSEs with transfected cells was significantly lower (50%) (Figure 6(b)) as compared to the HSEs with non-transfected cells. These findings confirm that the increased expression of LL-37 in the HSEs could be advantageous to prevent early bacterial colonization.

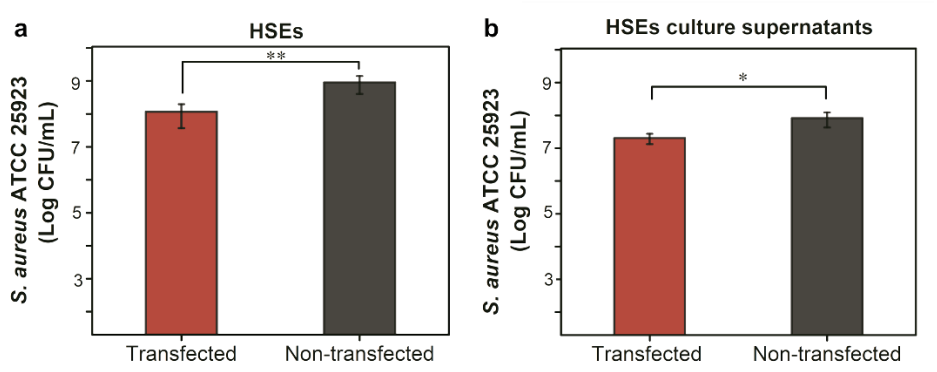

Figure 6. *In vitro* antibacterial efficacy of HSEs expressing LL-37 against planktonic *Staphylococcus aureus* ATCC 25923. (a) Transfected HSEs reduced the CFU/mL by one log compared to the non-transfected HSEs. (b) CFU/mL in the transfected HSEs culture medium. Co-cultures of HSEs and bacteria were incubated for 24 h at 37°C. Each data point represents the mean ± standard deviation (n = 3). One-way ANOVA followed by HSD test: *$p < 0.05$. **$p < 0.01$

DISCUSSION

As an alternative to the traditional antibiotics, gene therapy has allowed to genetically modify skin cells. Incorporated into biopolymer-based matrices they can secret in a sustained manner, proteins of interest such as the LL-37.[33] In this study, we describe a non-viral approach to increase the expression of the LL-37 peptide in HSEs generated with genetically modified human fibroblasts and keratinocytes by LPEI/pDNA polyplexes. Transfected HSEs express higher concentrations of LL-37 and possess characteristics close to native skin and exhibit improved antimicrobial activity against *S. aureus* strain *in vitro*.

Despite the fact that several studies have investigated the *in vitro* activity of LL-37 against two of the most common bacterial isolates in human wounds, *S. aureus* and *P. aeruginosa*, there are still discrepancies in the reported values for MIC and

MBC. Some of these concentrations previously reported in the literature are very high so that the combined use of antimicrobial peptides together with standard antibiotics has been proposed to achieve the killing efficacy.[34,35] However, the synergistic effect of LL-37 and an antibiotic can be explained by the amphipathic conformation of LL-37 that could mediate membrane permeabilization and facilitate the entry of the antibiotic into bacterial cells to achieve their goals.[36] In addition, the possible cytotoxicity of the combinations between antibiotics and antimicrobial peptides on human cells is not clear, whereas the use of LL -37 has shown positive effects on the proliferation and differentiation of various cell types, which could be a possible adjunct for regeneration.[37-39] Therefore, the application of LL-37 alone remains an attractive strategy, as it is less likely to induce resistance and mutagenesis in bacteria in the natural environment compared to antibiotics.[40]

In previous studies,[36,37] the attenuation of the antimicrobial activity of antimicrobial peptides has been observed when tested under conditions in which existing traditional antibiotics are active. This has been attributed to the inactivation of the structures of the cationic peptides by physiological concentrations of NaCl and divalent metal cations, among other variables.[41] For that reason, we have explored the manipulation of the bacterial culture medium including the addition of sodium bicarbonate, making them physiologically more relevant, and therewith decrease the MICs for the treatment of skin infections *in vivo*.

Our results demonstrated that at sub-MICs, starting at 16 μM, LL-37 was effective against *S. aureus* in the presence of sodium bicarbonate (pH 7.4) (Figure S2(a)). Moreover, LL-37 demonstrated to be bactericidal against *P. aeruginosa* at concentrations higher than 64 μM, regardless of the presence of sodium bicarbonate (Figure S2(b)). Moreover, LL-37 demonstrated to be bactericidal against *P. aeruginosa* at concentrations higher than 64 μM, regardless of the presence of sodium bicarbonate (Figure S2(b)). This concentration is higher compared to that of *S. aureus* possibly due to the activity of the extracellular DNA (eDNA) which is actively released or secreted by bacteria.[42] eDNA has been shown to bind metal cations like magnesium (Mg^{2+}). In *P. aeruginosa*, this is especially important, since it is known that the two-component regulatory systems, called PmrA-PmrB and PhoP-PhoQ, respond to the presence of limiting concentrations of Mg^{2+}, which regulates the expression of virulence genes that modifies lipopolysaccharides and contributes to survival against the activity of antimicrobial peptides.[43,44] Results for *S. aureus* seem

to be in line with those presented in literature where LL-37 was found to be less effective toward the Gram-positive strains cultured in normal bacterial medium (without sodium bicarbonate).[45,46] Sodium bicarbonate can contribute to the activity of antimicrobial peptides such as LL-37 through the alteration of the electrochemical gradient of protons, known as the proton motive force (PMF), generated through the bacterial membrane.[30] The proton motive force is necessary for the synthesis of ATP and active transport of molecules across the bacterial membrane.

It has been shown that LL-37 peptide is capable of interacting with several human cell types and influencing their behavior, improving cell migration and proliferation processes, and even promoting differentiation.[47,48] Significant advances have been achieved regarding the elucidation of the LL-37 effects over human cells such as neutrophil and monocytes. However, current knowledge regarding its effects on cells incorporated in biological matrices such as HSEs are largely unknown. In this study, we demonstrate that concentrations of LL-37 ranging from 2 μM to 256 μM were safe for the HSEs, and that concentrations up to 64 μM increased the metabolic activity after 7 and 14 days in culture (Figure 2(a)). These findings coincide with various previous literature reports showing that LL-37 is cytocompatible toward keratinocytes and fibroblasts from the skin and that it could also stimulate cell proliferation, mainly through its association to cell receptors like fibroblast growth factor receptor (FGFR) and epidermal growth factor receptor (EGFR).[13,49,50] In fact, we evaluated the number of cells in HSEs exposed to different concentrations of LL-37, specifically those previously established as MICs for *S. aureus* and *P. aeruginosa* (32 μM and 64 μM, respectively). The number of cells in the treated HSEs was higher compared to the negative controls (HSEs exposed to 0 μM LL-37) (Figure 3(b) and 3(c)). These findings suggest that possibly an increase in the production of LL-37 by transfected keratinocytes and fibroblasts could positively regulate their proliferation when cultured in HSE.

The use of matrices constructed with biomaterials such as fibrin, in conjunction with delivery systems such as polyplexes, has allowed the expression of the transgene to be maintained for a longer period of time and a more localized release of the protein.[51-53] In a previous study, the production of linear polyethylenimine polyplexes for non-viral transfection of human keratinocytes and fibroblasts was optimized, and resulted in a higher number of copies of the CAMP gene (encoding LL-37 peptide).[27] Using this optimized system gave a higher

expression of LL-37 in transfected cells when stimulated with bacterial culture supernatants compared to non-transfected cells (Figure 3). The stimulation could be explained due to the presence of products derived from the *S. aureus* cell wall such as peptidoglycans, polysaccharides, and lipoteichoic acids as well as proteins (e.g., the staphylococcal protein A) that are anchored to the cell wall envelope, and constitute critical factors for bacterial adherence to and invasion of host tissue.[54]

In addition, transfected HSEs expressed higher levels of LL-37 for up to 14 days in culture (Figure 4), although on day 21, LL-37 release was similar to controls. This is corresponding to the endogenous LL-37 expression by the keratinocytes of the human skin.[31,55] In fact, the LL-37 immunostaining was detected in the epidermal and dermal layers of the transfected HSEs, but this pattern of staining was only observed in the epidermal layer of non-transfected HSEs (Figure 5). Furthermore, transfected HSEs possess histological characteristics close to native human skin, demonstrating that the expression of LL-37 did not alter the architecture of the model.

LL-37 expression decreases considerably in chronic skin wounds, which could explain why these wounds are more predisposed to bacterial colonization and less efficient in healing.[56] Enhanced production of LL-37 in fibrin-based HSEs constructed with fibroblasts and keratinocytes transfected by polyplexes system could favor wound healing through its antimicrobial properties. A report showed that enhancing LL-37 production through its adenoviral expression in burn wounds in rats that genetically modified cells were more efficient in controlling infection than direct application of the peptide.[57] Moreover, there is evidence that LL-37 exhibits antimicrobial properties through its non-viral expression in the human keratinocyte progenitor cell line (NIKS) cultured in a full-thickness human skin substitute generated with type 1 collagen.[20] In this study, HSEs containing transfected cells were able to decrease bacterial growth of a planktonic strain of *S. aureus* after 24 h of co-culture, suggesting that the levels of LL-37 were sufficient to control bacterial colonization in the HSEs. Furthermore, as three-dimensional (3D) models, HSEs can be produced in different sizes in order to increase the population of cells expressing the protein of interest, which could more easily prevent bacterial attachment or early biofilm formation in the wound environment.

Therapeutic use of these genetically engineered skin models with improved antimicrobial activity could have important advantages compared to the use of antibiotics, due to the natural difficulty that microorganisms have to acquire resistance to host defense peptides.[13,58] In addition, LL-37 has been shown to enhance wound healing and vascularization by promoting endothelial proliferation and the chemotaxis of immune cells.[59] Thus, future studies could focus on the evaluation of processes such as angiogenesis and re-epithelialization of the wound.

In conclusion, our study demonstrates that HSEs built with cells that express higher levels of the LL- 37 antimicrobial peptide through the linear polyplexes system could be an efficient way to prevent bacterial colonization and therewith prevent infections in skin wounds.

AUTHOR DISCLOSURE

No competing financial interests exist.

FUNDING INFORMATION

This work was supported by the Abel Tasman Talent Program (ATTP) of the University of Groningen, and COLCIENCIAS National Doctoral program (code 727-2015).

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SUPPORTING INFORMATION

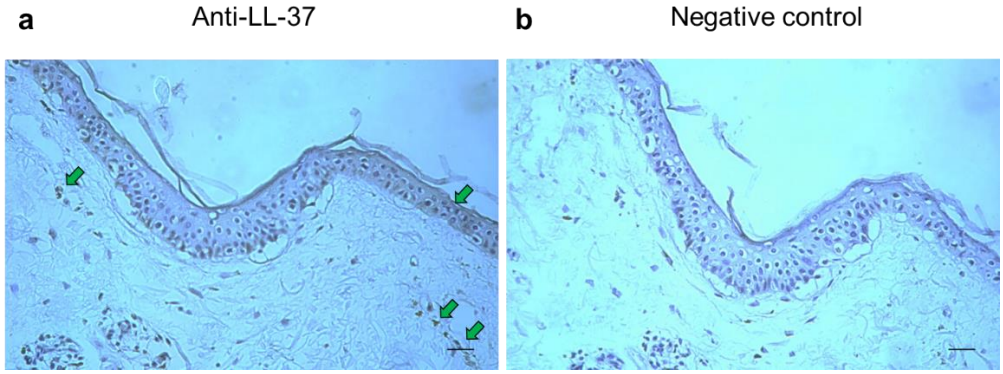

Figure S1: Negative control of transfected human skin equivalents (HSEs) for immunohistochemistry. (a) sections of transfected HSEs showing immunoreactivity for LL-37 (green head arrows). (b) sections of transfected HSEs used as negative control, treated with 1% BSA in PBS and without the primary antibody. As expected, none LL-37 immunostaining was detected.

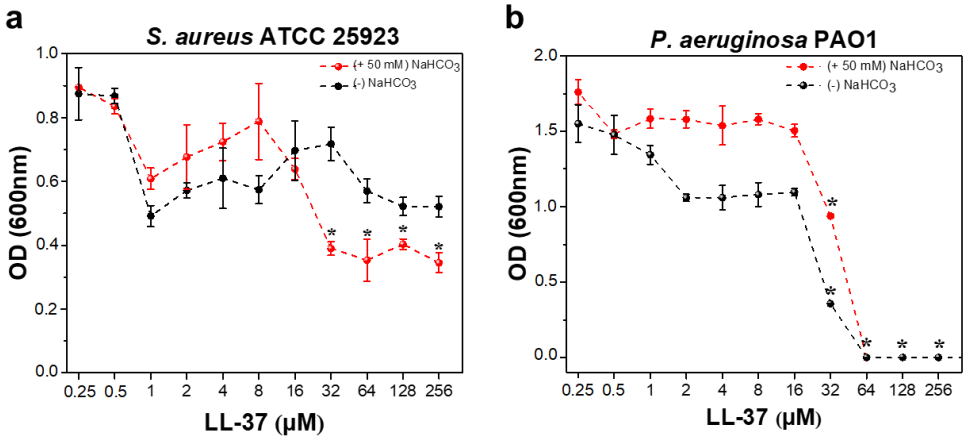

Figure S2: Planktonic growth evaluated in terms of the optical density (OD = 600 nm) of (a) *Staphylococcus aureus* ATCC 25923 and (b) *Pseudomonas aeruginosa* PAO1 after treatment with different concentrations of LL -37 peptide in the presence or absence of 50 mM $NaHCO_3$ at pH 7.0. Data represent the mean value ± standard errors. One-way ANOVA followed by Tukey's honestly significant difference (HSD) test. * $p < 0.05$, significant reduction compared to negative controls (0 μM LL-37). Three independent experiments were performed in triplicates.

Chapter 5

Viscoelastic Properties of Fibrin-agarose Hydrogels Dictate Favorable Fibroblast Response for Skin Tissue Engineering Applications

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Manuscript in preparation

ABSTRACT

Hydrogel-based skin equivalents are promising tissue engineering approaches for wound healing, due to their potential to mimic characteristics of the native extracellular matrix (ECM). In this regard, fibrin has been described as an ideal biomaterial for developing hydrogel scaffolds, because of its physiological role in wound healing and the ability to be remodeled by cells. However, the poor mechanical properties of fibrin hydrogels make them much weaker than native tissues, which limits their use in clinical practice. To overcome this limitation, fibrin-agarose (FA) hydrogels have been developed and successfully used for the preparation of different bioengineered tissues. Nevertheless, there is still a need to understand the viscoelastic properties of these FA-hydrogels in relation to agarose concentration and their effect on cell behavior and function. This study reports the characterization of the viscoelastic properties of FA-hydrogels prepared with different agarose concentrations (0%, 0.5%, 1%, 1.5%, and 2%), and their influence on the biological behavior of seeded human dermal fibroblasts. Cell-seeded and unseeded FA-hydrogels were tested by uniaxial compression on a low-load compression tester. The addition of agarose significantly correlated with mechanical and structural changes in FA-hydrogels. The stiffness, relaxation time constants 1 ($\tau 1$) and 2 ($\tau 2$), and fiber diameter increased with addition of agarose, whereas the porosity decreased. The main changes in cell behavior (metabolic activity, and cell proliferation) occurred in the early stages of culturing and were related to the displacement of fast ($\tau 1$) and intermediate ($\tau 2$) Maxwell elements. Subsequently, fibroblasts seeded in FA-hydrogels in concentrations lower than 1% agarose improved their proliferation and morphology after two weeks of culturing, which was demonstrated by the expression of F-actin (actin filaments) and the collagen deposition. Collectively, these results confirm that cell behavior in FA-hydrogels were strongly influenced by their viscoelastic properties, which in turn were coupled with the structural properties of the hydrogel. Therefore, viscoelasticity is a key parameter to consider when designing FA-hydrogels for tissue engineering application.

INTRODUCTION

Hydrogel-based skin equivalents have emerged as the most promising approaches for skin tissue engineering applications, due to their characteristics to mimic the native skin microenvironment by acquiring different shapes and incorporating cell populations.[1,2]

One of the biggest challenges facing the use of hydrogels for skin tissue engineering applications is the ability to replicate the mechanical characteristics of the skin, but also to get a robust hydrogel which can survive the stresses active on the skin.[1,3] As other soft tissues and extracellular matrices (ECMs), the skin exhibits a combination of elasticity and viscosity (viscoelasticity).[4,5] Elasticity is defined as the physical property of a substance that allows to change its volume, length, or shape in response to a force, followed by recovery to its initial configuration once the force is removed. The elasticity of the skin enables it to change and recover its shape when it is stretched or deformed. Viscoelasticity is related to adaptability of the ECM network and the water content of the skin which adds the principle of flow or viscosity. In response to a constant deformation, viscoelastic materials exhibit stress relaxation or time dependent strain increase i.e. creep in response to a constant stress.[6] The viscoelastic behavior of the skin provides protection against injury by allowing additional movement (as compared to pure elastic properties) of skin structures away from and returning toward baseline without breaking.[7]

Fibrin hydrogels are considered promising for skin tissue engineering applications because they are easily obtained from the patient's blood, making them a suitable alternative in clinical protocols of autologous use.[8,9] Furthermore, fibrin hydrogels offer additional advantages such as low cost, ease of availability, biocompatibility and the ability to be remodeled by cells and retain certain growth factors.[10,11] However, they are delicate and difficult to handle thus several studies have already focused on improving their mechanical strength, for example by combining fibrin with other natural polymers such as agarose, therewith forming an interpenetrating polymer network in which both polymers cross-link simultaneously.[12,13] Agarose has been commonly used in biomedical applications due to its self-gelling properties, high water content, and poro-elastic structure that together provide a microenvironment for cellular activity.[14,15] Moreover, its biomechanical properties can be adjusted by changing the concentration of agarose, resulting in flexible characteristics similar to the desired tissue. The potential of

fibrin-agarose interpenetrating polymer networks (FA-hydrogels) to produce compatible scaffold biomaterials has already been demonstrated with multiple tissue models such as the cornea,[16] oral mucosa,[17] and nerve tissue.[18] Nevertheless, there is limited knowledge about the effect of different concentrations of agarose on the viscoelastic behavior of FA-hydrogels and the resulting cellular response.

It is well established that fibrin hydrogels are viscoelastic, which implies that they exhibit both elastic and viscous characteristics when deformed.[19] With the addition of agarose, the relative degrees of these elastic and viscous properties will change and will determine the response of FA-hydrogels to the forces to which they are subjected (e.g., the mechanical deformation of the skin).[20] Furthermore, the agarose content can also modify the microstructural properties of hydrogels, and these changes in conjunction with the physical properties, are known to regulate the ability of a variety of cell types to spread, grow and proliferate in three-dimensional (3D) cultures.[6,21]

In this study, we investigated the viscoelastic properties of FA-hydrogels with different concentrations of agarose and determined the cellular response of human dermal fibroblasts to evaluate the potential use of these hydrogels for skin tissue engineering applications.

METHODS

Cell culture

Human Dermal Fibroblasts (NHDF-Ad) tested and certified as mycoplasma-free and virus-free (HIV-1, hepatitis B, and hepatitis C) were purchased from Lonza, The Netherlands, and cultured in 75-cm^2 culture flasks with Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher Scientific, USA) high glucose (4.5 g/l), supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco, USA) and 1% (v/v) penicillin-streptomycin. Cells were incubated at 37°C, and 5% CO_2 until they reached 80% confluency. Passages between 1 and 5 were used for all experiments.

Fibrin-agarose (FA) hydrogels preparation

For the preparation of the 3D FA-hydrogels, a solution (1 mL) of pooled human plasma, 140 μL NaCl (150 mM), 14 μL of tranexamic acid (5 mg/mL) (CAS Number 1197-18-8, Sigma), and 40 μL of a fibroblasts suspension ($2x10^5$ cells/ mL) was mixed with 0%, 0.5%, 1%, 1.5% or 2% (w/v) of agarose type VII (CAS 9012366, Sigma, USA). A stock solution of agarose was prepared of 5% (w/v) before

fibroblasts were added, for which the agarose was melted at 60°C in 50 mL NaCl (150 mM) and left overnight. Subsequently, the agarose stock solution was cooled to 37°C and kept until used. To promote fibrin polymerization, 140 µL $CaCl_2$ (1%) (Sigma) were added to the FA-hydrogel solution and gently homogenized. Then, 0.5 mL was poured into a 24-well plate (Corning, USA) and incubated in a humidified atmosphere with 5% CO_2, at 37°C for 30 min. FA-hydrogels with fibroblasts were cultured for 1, 7 and 14 days for the next experiments, and the culture medium was changed every three days.

Swelling ratio

FA-hydrogels without fibroblasts containing different concentrations of agarose (0%, 0.5%, 1%, 1.5% or 2% (w/v)) were prepared as described before in a 24-well tissue culture plate in a final volume of 0.5 mL. After 1 h at 37°C, they were removed from the wells and weighed to determine their "dry" mass (*Md*). Subsequently, the hydrogels were transferred to 400 µL phosphate buffered saline (PBS, 5 mM K_2HPO_4, 5 mM KH_2PO_4, 150 mM NaCl, pH 7.4), and left statically for 24 h at 37°C. The FA-hydrogels were weighed after wiping the excess PBS with tissue paper to obtain the "wet" mass (*Mw*), and the swelling capacity was quantified by means of its mass swelling ratio using Equation 1:[24]

$$\text{Mass swelling ratio} = \frac{Mw}{Md} \qquad [1]$$

FA-hydrogels viscoelasticity

The viscoelastic properties of FA-hydrogels with and without fibroblasts were determined using uniaxial compression on a low-load compression tester (LLCT).[22,23] FA-hydrogels of 2 mm thick were placed on a glass coverslip with double sided tape to prevent sample displacement. Each FA-hydrogel was compressed at a constant strain rate of 0.2 s^{-1} to 80% of its original thickness (strain, ε=0.2) with a 2.5 mm diameter steel plunger and kept compressed for 100 s. Strain (ε) was calculated as the change in thickness during compression normalized with the starting gel thickness. Force output from the LLCT was normalized by the area of cross-section of the plunger to get stress (σ). All data were analyzed with data fitting routine written in MatLab 2018 (MathWorks® Inc, Natick, USA). The elastic modulus was calculated as the slope of the stress-strain curve (Figure 1(a)) while the

stress relaxation was calculated by comparing initial stress (t = 0 s) versus final stress (t = 100 s) (Figure 1(c)).

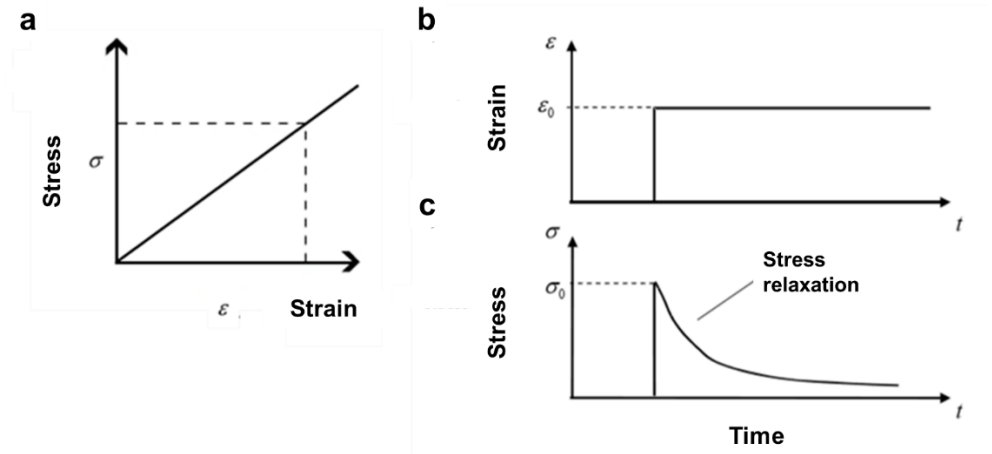


Figure 1. Elastic modulus and stress relaxation. (a) The elastic modulus is calculated as the slope of the stress-strain curve. (b) For a stress relaxation test, a constant strain is applied, and (c) the decrease (relaxation) in stress over time detected by the low-load compression tester.

During the stress relaxation, *E(t)* was defined as a time-dependent elastic modulus, where σ(t) is the measure of time-dependent stress (Figure 1c) and ε_o the constant strain value of 0.2 (Figure 1b), decreasing with time t during 100 s according to Equation 2:

$$E(t) = \frac{\sigma(t)}{\varepsilon_o} \qquad [2]$$

The measured stress relaxation curves were modeled using a generalized Maxwell model by fitting Equation 3:

$$E(t) = E_1 e^{-\frac{t}{\tau_1}} + E_2 e^{-\frac{t}{\tau_2}} + E_3 e^{-\frac{t}{\tau_3}} + \cdots E_i e^{-\frac{t}{\tau_i}} \qquad [3]$$

where $\tau_i = \eta_i / E_i$ is the relaxation time constant, η_i the viscosity and E_i the spring constant for each Maxwell element i. Using this formula, the number of Maxwell elements for each hydrogel could be calculated.

The optimal number of Maxwell elements required was determined using the Chi-square function following Equation 4:

$$x^2 = \sum_{j=0}^{100} \left[\frac{E_j - E(t_j)}{\sigma_j} \right] \qquad [4]$$

where j varies from 0 to 100 s, E_j is the measured value at time j, $E(t_j)$ is calculated from Eq. 3 and σ_j is the standard error of the device equal to 1.41 Pa.

Cell number and cell morphology

Cell number and cell morphology in FA-hydrogels were analyzed by fluorescence microscopy after 1, 7 and 14 days of incubation. To visualize the expression of filamentous actin (F-actin) and nuclei, FITC-labeled phalloidin and 4′, 6-diamidino-2-phenylindole (DAPI) (Sigma, D9564) were used, respectively. FA-hydrogels were first washed with PBS, fixed with 3.7% paraformaldehyde (Sigma) for 1 h, and subsequently washed with PBS three times. Afterwards, samples were permeabilized using 0.5% (v/v) Triton X-100 solution in PBS for 3 min and blocked with 5% bovine serum albumin in PBS for 30 min. FITC-labeled phalloidin (2 μg/mL) was applied to the samples followed by incubation for 30 min at room temperature in the dark. DAPI (4 μg/mL) was then added to counteract the cell nuclei and the samples were incubated for 1 h. After staining, FA-hydrogels were visualized with a fluorescence microscope (Leica DMR, Wetzlar, Germany), and ≥ 10 images per sample were taken for the cell counting. Three independent samples were analyzed per each agarose concentration.

Cell metabolic assay

Cell metabolic activity in the FA-hydrogels was determined using XTT ((2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazo-lium-5-carboxanilide salt), A8088 AppliChem, The Netherlands). Briefly, the FA-hydrogels were prepared as described above with an initial number of $2x10^5$ cells/mL. Similarly, FA-hydrogels without cells were prepared and used for background absorbance. After 1, 7 and 14 days in culture, a solution of 500 μL of XTT reagent (in a volume ratio of 1:50) was mixed with the growth medium present on top of each sample and incubated at 37°C, with 5% CO_2 for 6 h. Absorbances A460nm and A690nm were measured using a microplate reader (Shimadzu, Japan), and the metabolic activity of the cells seeded in the FA-hydrogels was calculated relative to the fibrin hydrogels at 0% agarose according to Equation 5.

$$\text{Metabolic activity (\%)} = \frac{A_{FA-hydrogel}\,460nm - A_{FA-hydrogel}\,690nm}{A_{0\%\,FA-hydrogel}\,460nm - A_{0\%\,FA-hydrogel}\,690nm} \text{ x } 100 \qquad [5]$$

The absorbance of all FA-hydrogels samples was corrected by subtracting the background absorbance of FA-hydrogels without cells.

Morphological characterization of FA-hydrogels

Morphology of FA-hydrogels with and without cells was characterized by scanning electron microscopy (Jeol JSM 6490 LV), under high vacuum to get high-resolution images. The samples were fixed in glutaraldehyde 3% in PBS (pH 7.4) followed by 1% electron microscopy grade osmium tetroxide for 90 min. Afterward, the samples were dehydrated in increasing concentrations of ethanol (30%, 50%, 70%, 90%, 95% and 99%), critical point dried at 31°C and 1072 psi, and sputter coated with gold (Denton Vacuum Desk IV). The obtained SEM images were analyzed using the software ImageJ. The percentage porosity was calculated with the "Analyze particles" features of this software. The area of the fibers was calculated in the FOV and subtracted from the total area in the FOV, which gave the porosity.

Histological analysis of collagen

FA-hydrogels seeded with fibroblasts ($2x10^5$ cells/mL) were fixed after 7 days in a 4% paraformaldehyde (Sigma, CAS no.30525-89-4) solution for 24 h at 4°C, then dehydrated through sequential ethanol (70–100%) treatment and embedded in paraffin blocks. Subsequently, histological sections of 5 μm in thickness were prepared using a microtome (Leica RM2265, Germany) and stained with Masson's trichrome to visualize the presence of collagen under an optical microscope (Leica DMR Leica, Wetzlar, Germany).

Statistical analysis

All data points are expressed as mean values ± standard deviation. Statistical analysis was performed with OriginPro 2018 (v9.5.1). Data were analyzed using one-way or two-way analysis of variance (ANOVA) with Tukey's test to determine differences between groups *$p< 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$, respectively. Correlations between the increase in agarose concentrations and the variables of viscoelasticity and cellular response were determined by Pearson's (r) coefficient. Values between 0.70 to 1 (-0.70 to -1) were considered high, and between 0.50 to 0.70 (-0.50 to -0.70) were considered moderate, positive (negative) correlations.

RESULTS

Swelling behavior of FA-hydrogels without fibroblasts

To investigate the swelling behavior, 0%, 0.5%, 1%, 1.5%, and 2% FA-hydrogels without cells were prepared, and swelled in PBS at 37°C for 24 h. As shown in Figure 2, the mass swelling ratio significantly decreased for 0% and 0.5% FA-hydrogels compared to concentrations higher than 1% agarose ($p < 0.001$). This can be explained by the intrinsic contraction of fibrin hydrogels, which are characterized by a relatively rapid contraction of approximately 30% during the first 24 h, until they reach a plateau of approximately 35% after 10 days.[25] On the contrary, no significant differences were found between the groups of FA-hydrogels at concentrations higher than 1%, and the swelling ratio remained approximately 1. This suggests that the hydrogels absorbed around 1 gram of solvent per gram of their dry mass. Furthermore, higher concentrations of agarose resulted in thicker gels after 24 h of incubation, whereas thinner gels were formed with the lower agarose concentration (0% and 0.5%).

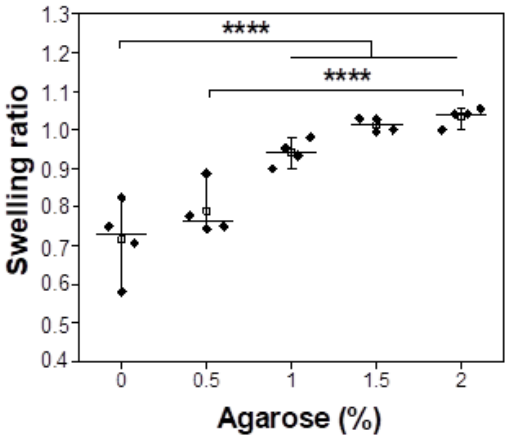


Figure 2. Swelling behavior of FA-hydrogels as a function of agarose (% w/v) concentration. The swelling ratio was assessed by immersing FA-hydrogels, dried for 1 h at 37°C, in PBS for 24 h at 37°C. Data show the mean values and the standard deviation (n=3). One-way ANOVA followed by Tukey's honestly significant difference (HSD) test: **** $p < 0.0001$.

Elastic modulus and stress relaxation of FA-hydrogels

The elastic modulus (stiffness) of 0%, 0.5%, 1%, 1.5%, and 2% FA-hydrogels with and without cells were evaluated after 1, 7 and 14 days in culture. The addition of agarose was directly correlated with an increase in the stiffness of the FA-hydrogels. In general, FA-hydrogels with and without fibroblasts showed an increase in stiffness with an increase in % agarose (Table 1). FA-hydrogels cultured with fibroblasts did not show differences in stiffness compared to hydrogels without cells. The stiffness of the FA-hydrogels with and without cells is decreasing with increasing timepoints. These results suggest that the stiffness of fibrin hydrogels is tunable by increasing the agarose concentration and this property remains unchanged regardless of the presence of cells.

After the FA-hydrogels were compressed with a constant strain value of 0.2 s^{-1} for 100 s, the decrease in stress was recorded as a function of time, and the time it took for each hydrogel to reach 50% stress relaxation was determined. No significant differences were found between FA-hydrogels, regardless of the presence of cells and culture time (Table S1). Taken together, these findings indicate that with increasing agarose concentration, the stiffness of the FA-hydrogels increases while the amount of stress relaxation is not affected.

Table 1: Elastic modulus (kPa) of FA-hydrogels with (CS) and without (US) fibroblasts at different agarose concentrations and evaluated after different timepoints.

Days	Agarose (%) in the FA-hydrogels									
	0%		0.5%		1%		1.5%		2%	
	CS	*US*	*CS*	*US*	*CS*	*US*	*CS*	*US*	*CS*	*US*
1	0.51 ± 0.03	0.64 ± 0.06	1.46 ± 0.23	1.51 ± 0.52	2.84 ± 1.02[a]	5.24 ± 0.80[a]	15.09 ±10.24[a]	14.40 ± 2.66[a]	13.78 ±7.26[a]	15.57 ± 7.06[a]
7	0.49 ± 0.19	0.99 ± 0.05	0.42 ± 0.19	0.96 ± 0.32	1.15 ± 0.30[a]	1.65 ± 0.21[a]	7.51 ± 1.51[a]	6.94 ± 2.05[a]	1.27 ± 0.24[a]	11.10 ± 0.82[a]
14	0.27 ± 0.04	0.76 ± 0.12	0.89 ± 0.24	0.62 ± 0.33	3.16 ± 0.73[a]	0.92 ± 0.02[a]	3.74 ± 0.86[a]	5.58 ± 2.85[a]	2.07 ± 0.55[a]	4.93 ± 3.32[a]

[a] indicates significance ($p < 0.001$) compared to 0% FA-hydrogels

Data represent the means ± standard deviation from three independent non-paired samples per timepoint. Two-way ANOVA followed by Tukey's HSD test.

Maxwell analysis of FA-hydrogels

FA-hydrogels required three Maxwell elements to describe their stress relaxation (Figure 3), which suggests that there is a different relaxation behavior of the hydrogels on three-time scales that were defined as fast, intermediate, and slow. There was no difference between the three Maxwell elements for the FA-hydrogels of day 1, 7 and 14, for both with or without cells.

To further evaluate the viscoelastic behavior of FA-hydrogels in response to increased agarose concentration, Pearson's correlation coefficients (r) of hydrogels with and without cells after 1, 7 and 14 days in culture were analyzed. Figure 4 shows a high positive correlation between the relaxation time constants 1 (τ1) and 2 (τ2), and the increase in agarose concentrations at all evaluated time points, while the relaxation time constant 3 (τ3) did not correlate with the agarose content (Figure 4 (c, f, i)).

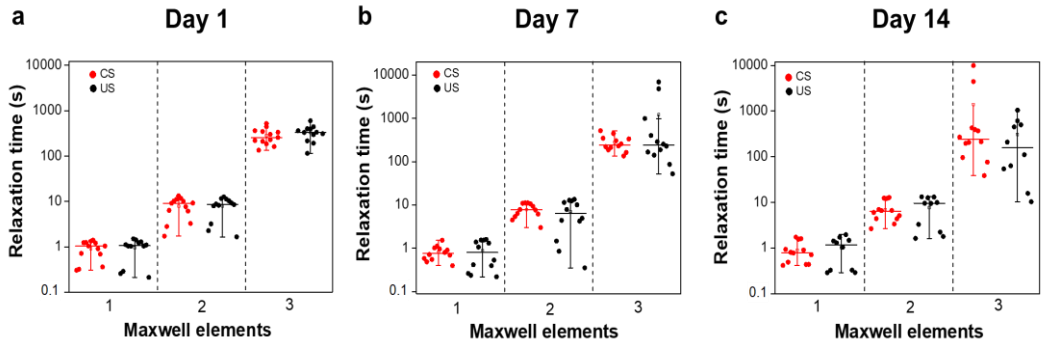


Figure 3. Relaxation time constants for the three Maxwell elements for FA-hydrogels, with cells (CS) and without cells (US) and different agarose concentrations. Note that no differences were observed between with and without cells. (a) 1 day, (b) 7 days, and (c) 14 days of incubation. Measurements were obtained from three independent non-paired samples per time point.

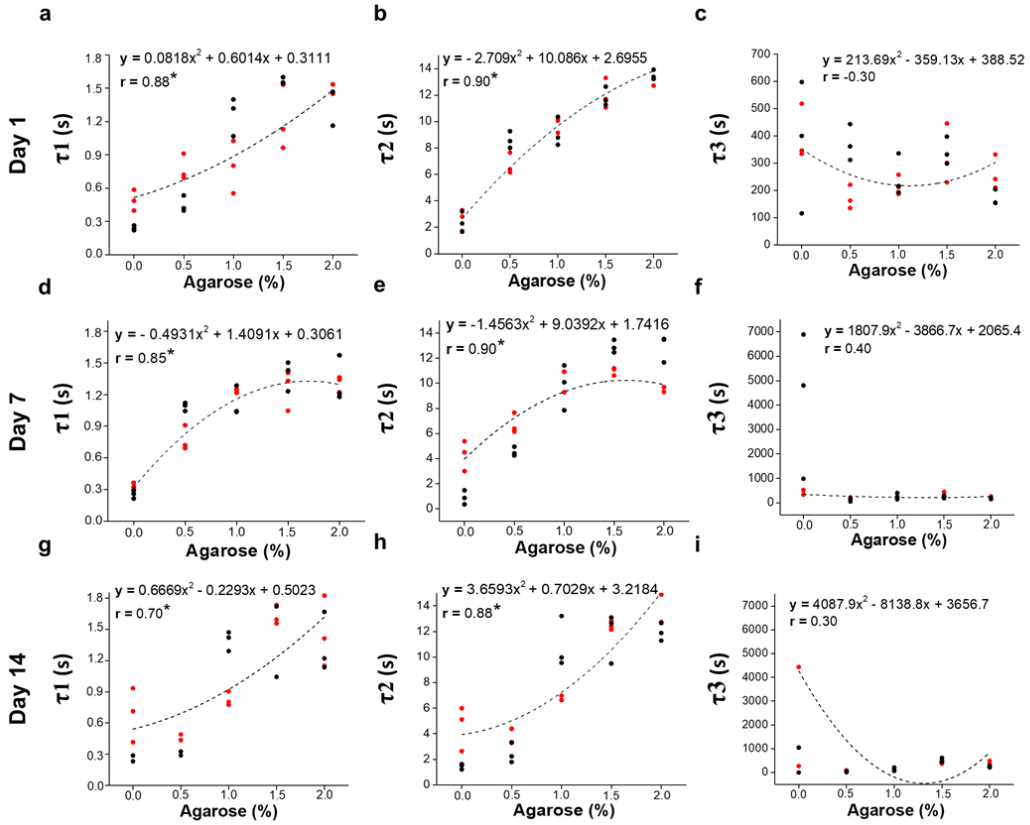


Figure 4. Correlations between the relaxation time constants 1 (τ1), 2 (τ2), and 3 (τ3) as a function of agarose concentrations after (a-c) 1 day, (d-f) 7 days and (g-i) 14 days of incubation. Note that the data with (red dots) and without cells (black dots) were analyzed in a single dataset since no differences were observed between them (see Figure 3).

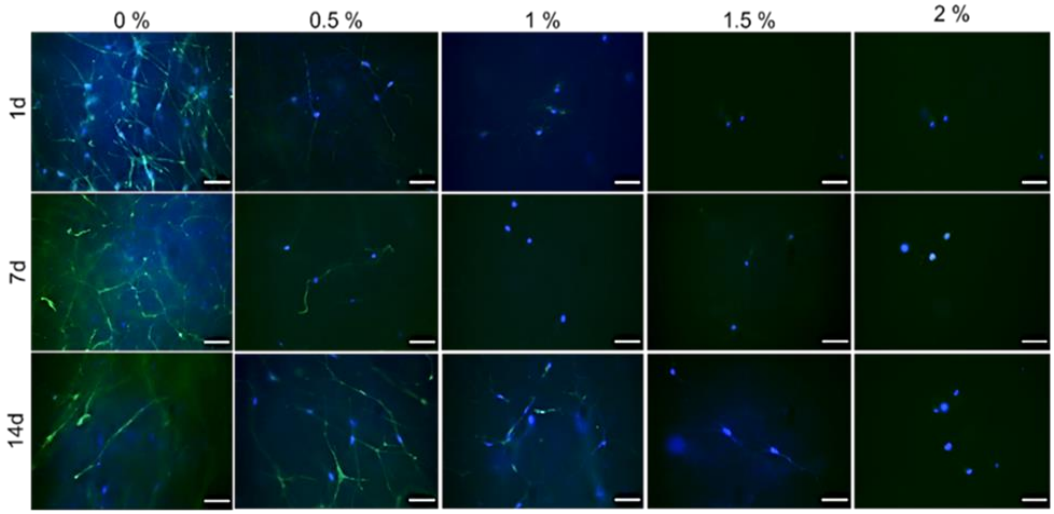
Fibroblasts response to FA-hydrogels

Interaction between cells and the hydrogel matrix is important for initial cell adhesion and proliferation, which are required when scaffolding for skin tissue regeneration. Cellular response to FA-hydrogels was first assessed by fluorescence microscopy after 1, 7, and 14 days of culture. Figure 5(a) shows the expression of F-actin (green filamentous extensions) in FA-hydrogels at different concentrations of agarose. The typical spindle-shaped morphology of the fibroblast was observed initially in the 0% and 0.5% FA-hydrogels. After 14 days in culture, cells seeded in

1% FA-hydrogels also exhibited this cell morphology. The higher cell density (blue dots) indicated by DAPI stain was observed in 0% and FA-hydrogels at concentrations ≤ 1% agarose after 14 days in culture. On the contrary, the cross-linked fibrin hydrogels with concentrations ≥ 1.5% agarose resulted in an important decrease of the F-actine expression, as well as the DAPI-stained nuclei.

The number of cells was significantly lower ($p \leq 0.001$) in 1.5% and 2% FA-hydrogels after 7 and 14 days of culture when compared to 0% (Figure 5 (b)), while the number of cells in the 0.5% and 1% FA-hydrogels increased significantly across the incubation time. Therefore, it is suggested that lower concentrations of agarose are more favorable for cell attachment and proliferation.

a



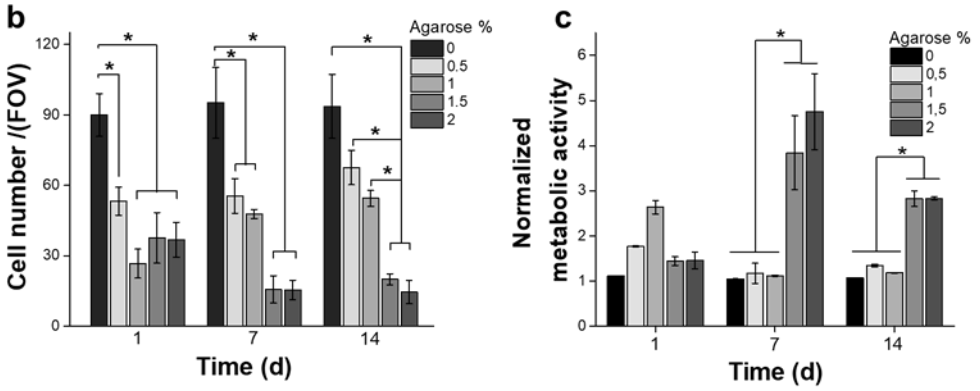


Figure 5. Fibroblasts response to FA-hydrogels with different concentrations of agarose. (a) Representative fluorescence images of DAPI and F-actin staining of cells within FA-hydrogels at 0%, 0.5%, 1%, 1.5% and 2% (w/v) agarose. Images were taken on days 1, 7, and 14 of culturing fibroblasts in FA-hydrogels. Blue color represents the cell nucleus and green color represents the cell cytoskeletal by the F-actin filaments. Scale bar denotes 50 μm. (b) Number of cells in FA-hydrogels evaluated by counting the DAPI-stained cell nuclei. A total of 10 fields of view (FOV) for each group were analyzed. (c) Normalized metabolic activity per fibroblast in FA-hydrogels with different agarose concentrations after 1, 7 and 14 days of culturing. The metabolic activity was normalized to 0% agarose. Data represent the means ± standard deviation (n = 3), Two-way ANOVA followed by Tukey's HSD test: * $p < 0.05$.

Cell metabolic activity in FA-hydrogels

Although the increase in agarose concentrations significantly decreased the number of cells within FA-hydrogels, this increase in agarose concentration did not affect the cellular metabolic activity. As shown in Figure 5(c), the normalized cellular metabolic activity per cell at concentrations ≤ 1% agarose did not show significant differences throughout the incubation period. On the contrary, a significant increase in cellular metabolic activity was observed in the highest concentrations of agarose (1.5% and 2%) on days 7 and 14 of culturing. Upregulation of the metabolic activity by cells can be due to the challenge the cells experience to survive under adverse conditions such as low availability of nutrients or hypoxia.

Microstructure analysis of FA-hydrogels

The SEM micrographs of FA-hydrogels prepared with different concentrations of agarose (0%, 0.5%, 1%, and 1.5%) were obtained and analyzed to assess the fiber diameter and the porosity in the presence or absence of cells cultured

for 24 h (Figure 6). The 2% FA-hydrogels were brittle and fractured, therefore evaluation by SEM was not possible. Figure 6 (b1) show the typical matrix of fibrin gels forming a uniform porous and fibrous scaffold composed of fibers with a diameter distribution up to 140 nm (Figure S3 (a)). In general, the evaluated agarose concentrations resulted in a significant increase of fiber diameter with less porosity (Figure 6(c)) when compared to the structure of 0% FA-hydrogels, regardless of the presence or absence of cells. In particular, 0.5% FA-hydrogels have high interconnectivity between fibers similar to that observed in 0% (Figure 6(b2)) but with a higher diameter of the fibers in the range of 150-200 nm (Figure S3 (b)). Figures 6(b3) and 6(b4) show that 1% and 1.5% FA-hydrogel networks were covered by the presence of agarose spots throughout the gel.

Cells cultured both in the 0% and 0.5% FA-hydrogels showed the typical spindle-shaped morphology of fibroblasts and were embedded and intimately adhered to the fibril network (Figure 6(a1) and 6(a2)). On the other hand, 1% and 1.5% FA-hydrogels showed the greatest changes in the hydrogel structure once seeded (Figure 6(a3) and 6(a4)), the cells appeared to be considerably less healthy despite the fact that they managed to penetrate the less porous network. These findings coincide with the fluorescent micrographs and the number of cells per FOV after 1 day of culture (Figures 5(a) and 5(b)).

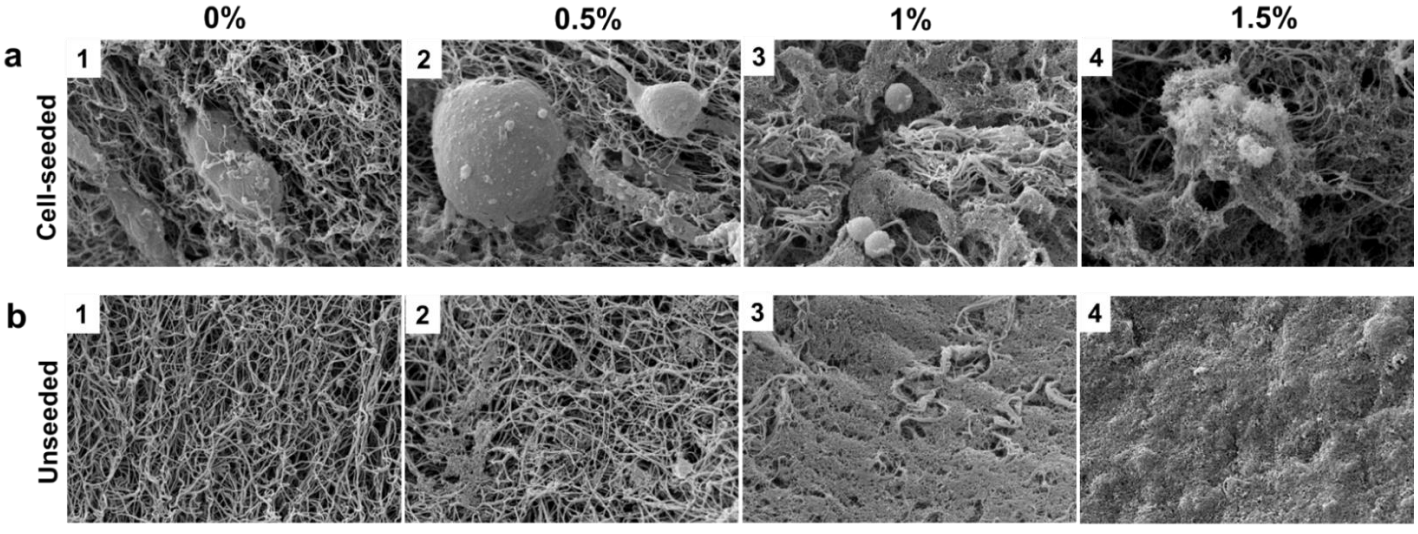

0%
0.5%
1%
1.5%
a
Cell-seeded
b
Unseeded
1
2
3
4
c
y = 13.399x² - 28.978x + 21.98
r = -0.90*
Porosity (%)
Agarose (%)
0
5
10
15
20
25
30
0.0
0.5
1.0
1.5

Figure 6. Representative SEM micrographs showing the microstructure of FA-hydrogels at different concentrations of agarose (0%, 0.5%, 1%, and 1.5%) after 1 day of culturing with fibroblasts (a1- a4), and FA-hydrogels without cells (unseeded) (b1 - b4). Scale bars represent 5 μm. (c) Correlation between porosity (%) of FA-hydrogels as a function of increasing concentrations of agarose after1 day.

Histological analysis of collagen deposition

The presence of collagen was evaluated by Masson's Trichrome stain in FA-hydrogels and native skin as positive control (Figure 7). Figures 7(a), 7(b), and 7(c) shows that 0%, 0.5%, and 1% FA-hydrogels had some staining for collagen (blue regions, red arrows). The 0% and 0.5% FA-hydrogels showed denser cell regions (black arrows) and collagen density similar to the dermal layer of native skin (Figure 7 (e)). In the 1% FA-hydrogels, collagen regions were also observed, although with a lower cell density, while in the 1.5% hydrogels only some cells were stained and collagen was hardly visible.

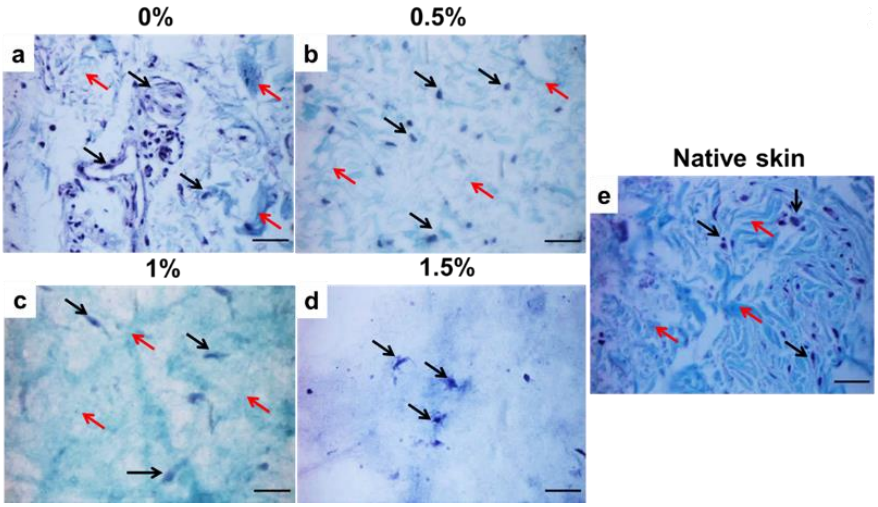


Figure 7. Histology of FA-hydrogels (a-d) and native skin (e) displaying the presence of collagen by the Masson's Trichrome stain (blue). Collagen areas (red arrows), and cellular populated areas (black arrows). Scale bars = 50 μm.

Correlations between the viscoelastic properties of FA-hydrogels and cell behavior

The addition of agarose was correlated with mechanical and structural changes in FA-hydrogels (Table 2). The stiffness, relaxation time constants 1 and 2, and fiber diameter increased with the addition of agarose, while metabolic activity, cell number and porosity decreased (Table 2). The viscoelastic behavior of the FA-hydrogels in response to the addition of agarose showed a negative but stronger correlation with the cellular parameters of proliferation and metabolic activity at all times evaluated (Figure S1 and Figure S2), which suggests that cell behavior is influenced by the viscoelastic properties of FA-hydrogels, mainly by the displacement of fast (τ1) and intermediate (τ2) Maxwell elements.

Furthermore, the low porosity by the addition of high concentrations of agarose (≥ 1.5%) also seemed to influence the cell number within the hydrogels (Table 2).

Table 2. Pearson's correlation coefficients showing the relationship between the addition of agarose (%), the viscoelastic and microstructural properties of the FA hydrogels, and the cellular responses.

Variables	Agarose (%)	Metabolic activity (%)	Cell number/ (FOV)	Porosity (%) (SEM)
Stiffness (KPa, 0.2 s^{-1})	0.65*	-0.44	-0.44	-0.43
Metabolic activity (%)	-0.87**	1	0.80**	0.46
Cell number/ (FOV)	-0.90**	0.80**	1	0.76**
Porosity % (SEM)	-0.91**	0.46	0.76**	1
Fibre diameter (nm) (SEM)	0.67*	-0.64*	-0.68*	-0.28
τ_1	0.84**	-0.69*	-0.73**	-0.86**
τ_2	0.90**	-0.68*	-0.65*	-0.83**
τ_3	-0.36	0.31	0.36	0.42

** High 0.70 to 1 (or -0.70 to -1) and * moderate 0.50 to 0.70 (or -0.50 to -0.70) correlation.

DISCUSSION

In this study, we characterized the viscoelastic properties and human dermal fibroblasts response to fibrin-agarose (FA) hydrogels produced with varying concentrations of agarose. The elastic modulus and stress relaxation properties, together with the microstructural pattern and cytocompatibility of human dermal fibroblasts, were investigated to determine the potential use of these hydrogels for skin tissue engineering applications.

Viscoelasticity[8] is accepted to be a near-universal property of living tissues and extracellular matrices (ECMs). A thorough understanding of this behavior is important to correctly design scaffolds for engineering functional tissue. In the context of skin tissue engineering, fibrin hydrogels are widely employed due to their biological and viscoelastic properties.[27] The viscoelastic behavior of the fibrin hydrogels (0% FA-hydrogels) produced in this study showed an instantaneous elastic behavior under uniaxial compression followed by time-dependent energy dissipation (i.e. stress relaxation) (Tables 1 and 2). Once the stress was removed, it was visually noticeable that the 0% FA-hydrogels remained partially deformed, suggesting that they were unable to maintain their mechanical stability possibly due to irreversible deformation of the fibrin network. Such irreversible deformation indicates that fibrin hydrogels are also viscoplastic.[28] Our observations are in line with the literature, as fibrin is regarded as a self-repairing polymer that can be reshaped (plastically deformed) under stress.[19] In absence of agarose, fibrin microstructural reorganization could occur quickly, resulting in fast relaxation rates. This viscoelastic and viscoplastic[26] behavior was observed in the 0% FA-hydrogels, in which 50% of the stress relaxation was achieved in a time of 55.1 ± 2.8 s (measured in a period of 100 s) (Table S1). In a previous study fibrin hydrogels showed strain-enhanced stress relaxation, as well as a substantial decline of strain that begins in 1 s, and almost disappears at 100 s.[29] The reason behind quick and irreversible rearrangement of fibrin to relieve stress is related to the knobs-into-holes bonds that hold fibrin together, which can break and then re-form in a different location.[27,30,31] Some of the common ECM formulations used for cell culture, such as type-1 collagen gels and the reconstituted basement membrane also exhibit viscoelastic and viscoplastic responses, unless they are sufficiently covalently cross-linked e.g. by combination with other more mechanically stable polymers,[29] or through the use of enzymes such as lysyl oxidase,[32] or the cellular transglutaminase, factor XIII-A.[33]

At first glance, agarose addition to fibrin did not affect the overall viscoelastic properties, since the stress relaxation time among hydrogels remained the same, regardless of agarose concentration (Table 2). However, Maxwell modelling of the stress relaxation behavior of the FA-hydrogels shows a strong positive relation between the agarose concentration and the relaxation time constants 1 and 2 (τ_1 and τ_2) (Figure 4 and Table 2). Increasing the agarose concentration led to a reduction in porosity (Figure 6(c)), with no pores visible in concentrations higher than 1%, according to SEM´s micrographs (Figure 6(b3-b4)). The decreased porosity would reduce fluid permeability, slowing the movement of water within the network under stress, leading to an increase in both τ_1 and τ_2 with addition of agarose, according to the Maxwell analysis.

Viscoelasticity is caused by various molecular mechanisms, including weak bond breaking in matrices, and fluid movement through a porous matrix. Since hydrogels consist mainly of water, we evaluated the influence of agarose concentrations on the swelling capacity of FA-hydrogels and their relationship with the porosity of the network. The 0% FA-hydrogels showed a deswelling behavior that has been previously reported,[25] and which is due to the typical contraction of these hydrogels due to protein release. A significant increase in the swelling ratio of FA-hydrogels was observed at concentrations higher than 1% agarose compared to 0% FA-hydrogels (Figure 2). This was in line with what was expected since agarose attracts water, which in turn provides greater resistance to volume loss. Previously it was observed that a 0.1% FA-hydrogel was 1.5 times thicker and a 0.5% FA-hydrogel was almost twice as thick as a hydrogel made from fibrin alone.[35]

The viscoelastic properties that were influenced by the increase in agarose concentrations correlate strongly with lower cell proliferation and metabolism (Figure S1 and Figure S2). The highest negative correlations were observed between the relaxation time constants (both τ_1 and τ_2) and cellular metabolic activity, as well as the number of cells after 1 day of incubation (Figure S1 (a, d) and Figure S2 (a, d)). This suggests that the main changes in cell behavior occurred in the early stages of culturing and were related to the displacement of fast (τ_1) and intermediate (τ_2) Maxwell elements.

Several studies have shown that the cell number is highest in porous hydrogels and show proliferation peaks on day 1 of cultivation,[36,37] which is also what is observed in our results (Figure 5(a)). On the other hand, pore size is known to be a key regulator of mechanical confinement in three-dimensional cultures, as smaller, rigid pores block migration because cells are unable to squeeze their nuclei through them[6,38] (Figure 6). This was evidenced by the negative staining for actin filaments in FA-hydrogels seeded at 1.5% and 2% agarose (Figure 5(a)). At these high concentrations, degradation of the hydrogel matrix would be required by the cells to overcome confinement and give them the possibility to migrate.[40]

Interestingly, recovery of cell morphology was observed after two weeks of culturing the fibroblasts seeded in FA-hydrogels at concentrations lower than 1% agarose. As shown in Figure 5a, the expression of F-actin (actin filaments) increased and was comparable to that observed at 0% agarose. In addition, a significant increase in the number of cells was observed at 0.5% and 1% agarose while the cellular metabolic activity did not show differences. These findings could also be associated with the ability of fibroblast and other cells to remodel their surrounding ECM either with deposition or degradation.[41] The ECM is a highly dynamic structure, which is constantly subjected to a remodeling process where ECM components are deposited or degraded by the embedded cells. ECM dynamics is essential as it allows the restructuring of tissue architecture. This corresponds with the high amount of collagen production by the fibroblasts we noticed for 0%, 0.5%, and 1% FA-hydrogels, which is also observed in the native skin (Figure 7).

The cell-matrix interaction together with the optimization of the mechanical properties makes FA-hydrogels an interesting approach for tissue engineering applications. Since the addition of agarose increased the stiffness of the hydrogels and avoided contraction of the matrix, which could favor its future clinical use since contraction is considered an important limitation for the manipulation, transport, and implantation of the hydrogel.[25] On the other hand, estimates of stiffness in tissues such as skin range between 4.5 kPa and 8 kPa,[42] which is similar to the results obtained for FA-hydrogels at concentrations greater than 1%. Soft hydrogels with an initial stiffness of around 1 kPa have been shown to fail to maintain their structural integrity after implantation.[43] Therefore, FA-hydrogels with concentrations of 1% are potential candidates for clinical applications, because they are strong, offer better cytocompatibility compared to hydrogels with higher concentrations of agarose.

In summary, our results confirm that both cell behavior and cell-ECM interactions in FA-hydrogels are strongly influenced by their viscoelastic properties, which in turn are coupled with the structural properties of the hydrogel, of which porosity seems to be the most important one. Therefore, viscoelasticity is a key parameter to consider for the design of FA-hydrogels as skin tissue engineering biomaterials. Furthermore, addition of 1% agarose in our opinion will be optimal from the point-of-view of robustness of the gel and simultaneously allowing for fibroblast proliferation and spreading.

ACKNOWLEDGMENTS

The authors acknowledge the support of Gert ten Brink in the preliminary SEM imaging

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

FUNDING

This work was supported by the Abel Tasman Talent Program (ATTP) of the University of Groningen, and COLCIENCIAS National Doctoral program (code 727-2015). F.D.M.G acknowledges the support of the Mexican National Council of Science and Technology (CONACyT; CVU-695528).

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SUPPORTING INFORMATION

Table S1: Time (s) of FA-hydrogels with (CS) and without (US) fibroblasts to reach 50% stress relaxation evaluated after different timepoints of incubating the gels at 37°C and 5% CO_2.

Days	Agarose (%) in the FA-hydrogels									
	0%		0.5%		1%		1.5%		2%	
	CS	*US*	*CS*	*US*	*CS*	*US*	*CS*	*US*	*CS*	*US*
1	58 ± 4	55 ± 3	55 ± 1	62 ± 4	57 ± 2	57 ± 4	66 ± 4	64 ± 2	66 ± 3	62 ± 2
7	51 ± 2	70 ± 11	50 ± 0	52 ± 1	61 ± 8	60 ± 6	61 ± 4	60 ± 3	59 ± 0	61 ± 2
14	64 ± 4	87 ± 18	52 ± 1	50 ± 0	54 ± 1	60 ± 9	69 ± 3	67 ± 0	75 ± 1	59 ± 1

Data represent the means ± standard deviation from three independent non-paired samples per timepoint.

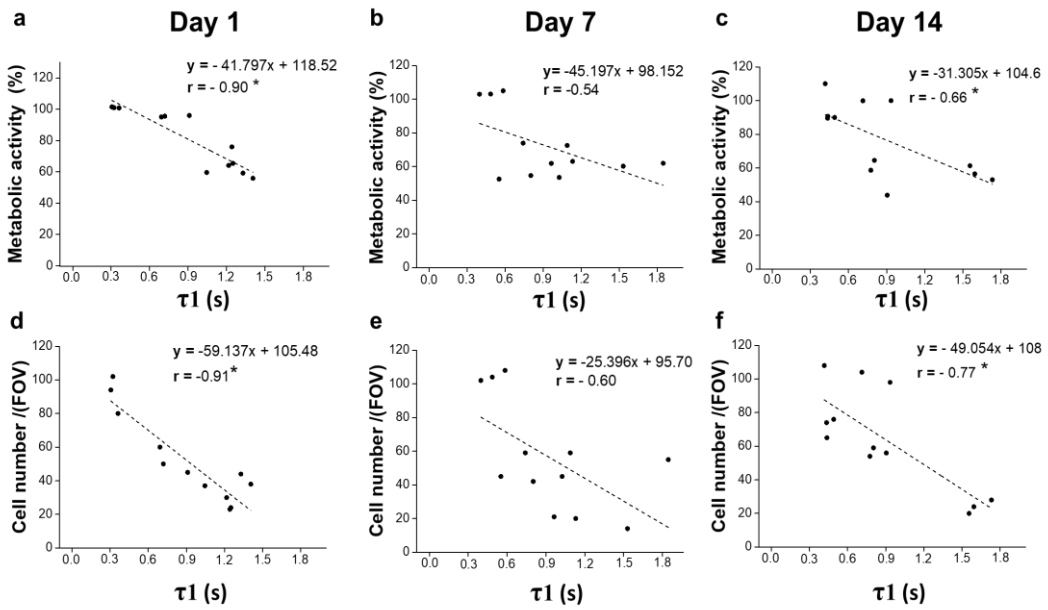

Figure S1. Correlation between (a-c) metabolic activity (%) of fibroblasts as a function of the Maxwell element relaxation time constant 1 (s) (τ1) of FA-hydrogels with different agarose concentrations cultured for different time points and (d-f) the same as a-c but now for cell number per FOV.

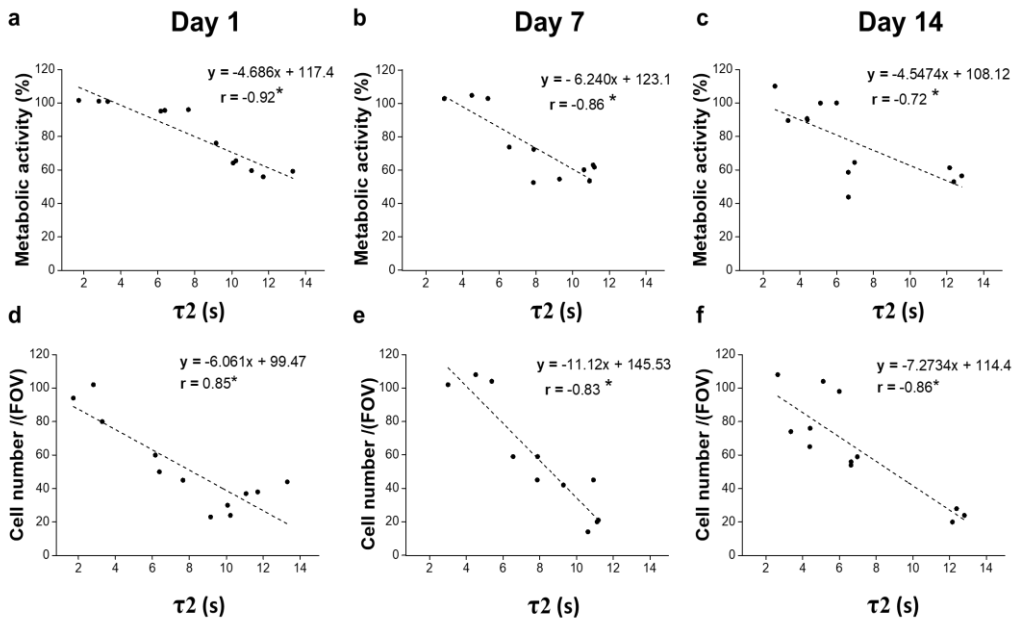

Figure S2. Correlation between (a-c) the metabolic activity (%) of fibroblasts as a function of the Maxwell element relaxation time constant 2 (s) (τ2) of FA-hydrogels with different agarose concentrations cultured for different time points and (d-f) the same as a-c but now for cell number per FOV.

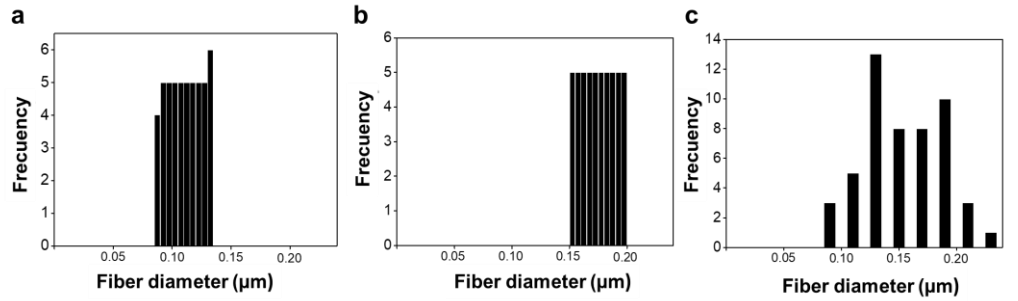

Figure S3: Fiber diameter distribution of FA-hydrogels determined from SEM images (Figure 6b). (a) 0% agarose, (b) 0.5% agarose, (c) 1% agarose. The frequencies of fiber diameters were analyzed using ImageJ on two independent samples (n = 50 fibers).

Chapter 6

General discussion

Skin wound infections with antimicrobial-resistant bacteria are considered one of the greatest threats to public health worldwide.[1] The rapid emergence of antibiotic-resistant bacteria has been attributed to the adaptation of these microorganisms to hostile environmental conditions, as well as the overuse and misuse of antibiotics.[2,3] As a result, skin wound infections are highly challenging to treat, often resulting in morbidity and mortality due to the development of septic processes in the patients.[4,5] Novel approaches on a non-antibiotic basis are therefore urgently needed to fight bacterial resistance and prevent infections in skin wounds.[6] This thesis focuses on the development and validation of a fibrin-based human skin equivalent (HSE) with overexpression of the antimicrobial peptide LL-37, and the optimization of their mechanical properties for skin tissue engineering applications.

THE CHOICE OF THE GENE DELIVERY SYSTEM

As a first step, we needed to choose a gene delivery system that would allow the genetic modification of primary keratinocytes and fibroblasts with the precursor gene that leads to LL-37 production (i.e. *CAMP* gene). It has been described that the success of gene therapy is largely dependent on the development of a vector or carrier that can efficiently deliver a gene to target cells with minimal toxicity.[7] The safety concerns regarding the use of viruses in humans make nonviral delivery systems a more attractive alternative, as they have low immunogenicity, and are easy and safe to prepare and use (chapter 1).[8] Two widely used systems for nonviral gene delivery are polyplexes and nucleofection.[9,10] Nucleofection toxicity in human keratinocytes has been previously reported,[9] with results that showed a decrease in cell size and with only 20%-42% of proliferative cells. This coincided with our findings in primary keratinocytes, since nucleofection led to a significant decrease in cell viability, although with higher transfection efficiencies in both keratinocytes and fibroblasts (chapter 2). Decreasing the cytotoxicity of nucleofection on keratinocytes implies optimization of the buffers purchased from the manufacturer, which in addition to increasing costs is quite challenging since information on the components of all solutions is lacking.[11] The polyplexes, on the other hand, proved to be efficient enough to achieve a high number of copies of the *CAMP* gene with low cellular toxicity. Furthermore, polyplexes can be prepared in a few steps using inexpensive reagents and can be stored for a longer time than nucleofection solutions.[12] This cost-benefit ratio is especially important when conducting research in developing countries.

Transfection efficiency is commonly considered as the key factor when comparing different transfection methods. However, few studies consider the influence of the sequence to be introduced into the target cells, which in most cases is a plasmid vector. Our plasmid vector contained an internal ribosome entry site (IRES) to allow separate expression of the reporter gene (red fluorescent protein) and the *CAMP* gene in target cells, since the expression product of *CAMP* (i.e., LL-37) is metabolized extracellularly. We demonstrated that the expression of the reporter gene located downstream of the IRES sequence and whose translation was IRES-dependent was reduced compared to the absence of IRES (chapter 2). Similar results were described in previous studies carried out in bicistronic plasmids, [13] which, like ours, contained two distinct genes within one vector. Nevertheless, most recent studies found that gene expression under the control of multiple IRES elements has no effect on the posttranscriptional regulation in multicistronic plasmids, and that the fluorescence output of reporter gene is proportional to the number of IRES repeats.[14,15] Therefore, care should be taken regarding the construction of the plasmid vector, as well as in determining which gene should be positioned as the first or second gene in a bicistronic construct. In our case, the *CAMP* sequence was located upstream of IRES, so its translation was IRES-independent.

Based on our findings, the polyplexes system was chosen for the genetic modification of skin cells in this work. Since the construction of HSEs suitable for tissue engineering applications must start from healthy cultures,[16] mainly healthy keratinocytes capable of proliferating and forming stratified epithelia similar to native skin, and capable to produce larger amounts of the LL-37 peptide together with fibroblasts.

LINEAR POLY(ETHYLENIMINE) (LPEI): A BETTER ALTERNATIVE FOR SKIN CELL TRANSFECTION.

Branched and linear forms of poly(ethylenimine) (BPEI and LPEI, respectively) have been commonly used as gene therapy delivery agents.[17] The success of these polymers in gene delivery strictly depends on the kind of target cells, and until now, their behavior in the transfection of primary fibroblasts and keratinocytes had not been described (chapter 3). BPEI and LPEI in different weight concentrations were varied for evaluating the formation and colloidal characteristics of the polyplexes. The PEI based polyplexes showing diameters between 250 and

450 nm and a positive surface charge (+30 ± 2 mV) at a Nitrogen/Phosphate ratio of 19, show the desired characteristics for interaction with the cell membranes and subsequent endocytosis.[18] This positive surface charge confers electrostatic stability to the solution and prevents polyplexes aggregation since it allows sufficient repulsive forces between them.[19]

Several studies have reported that the transfection efficiency of LPEI polyplexes *in vitro* was greater than that of BPEI polyplexes.[20] They also proposed that this behavior might be a result of a less strong conjugation of LPEI with the DNA. In transfected keratinocytes and fibroblasts, both LPEI and BPEI allowed the expression of the reporter gene and increased the *CAMP* gene expression. However, LPEI showed superior performance as it led to higher transfection efficiencies with the highest cell viabilities compared to BPEI. This is due to the fact that LPEI formed less compacted polyplexes than BPEI, so it is possible that they can dissociate easily within cells and release DNA, while polymer residues are degraded or diluted by exocytosis processes.[21]

These findings contribute to a broader knowledge of the effects of polyplexes system on the genetic modification of skin cells and allowed to establish the optimal transfection conditions for both human keratinocytes and fibroblasts.

THE GENERATION OF HSEs WITH ANTIMICROBIAL POTENTIAL

Even though various human skin substitutes are commercially available (chapter 1), to date no dermo-epidermal model for the prevention of skin wound infections has been reported. In this work, we developed fibrin-based HSEs seeded with primary fibroblasts and keratinocytes previously transfected through LPEI polyplexes (chapter 4). Prior to the construction of the HSEs, quantification of the LL-37 peptide in monolayer cultures of transfected cells showed a low production of the peptide despite the fact that these cells expressed high copies of the *CAMP* gene. This result was initially considered contradictory since it was expected that the increase in the number of copies of the gene was accompanied by equal changes in the encoded proteins. However, this is not always the case. In fact, how cells change the expression of proteins in response to their environment is one of the most fundamental questions in biology. The multitude of steps between transcription and translation provides many different regulatory opportunities and always leaves a "door open" for future research questions.[22,23]

Taking this into account, we challenged the transfected cells through stimulation with metabolites derived from bacteria, obtaining a significant increase in the LL-37 peptide production compared to the non-transfected cells. The increase in LL-37 levels was also observed in HSEs constructed with these modified cells and remained elevated up to 14 days in culture. Furthermore, HSEs containing transfected cells were able to decrease bacterial growth of a planktonic strain of *S. aureus* after 24 hours of co-culture.

The increased secretion of the LL-37 peptide after the transfected HSEs were exposed to metabolites from bacteria correspond with what has been reported about the expression of this peptide on epithelial surfaces, especially for keratinocytes in airways or skin.[24] Under normal conditions, the *CAMP* gene product is directed to the storage granules of cells and can be stimulated by both exogenous microbial components or endogenous signal molecules such as lipopolysaccharides or bacterial DNA.

In this sense, the lack of stimulation could explain the results obtained in the *in vitro* antimicrobial activity tests when the LPEI/pDNA and BPEI/pDNA polyplexes were compared (chapter 3). It was observed that supernatants from the cells transfected with BPEI did not show activity against the evaluated bacterial strain, despite the fact that the expression of the *CAMP* gene had previously been confirmed. But, based on the findings in chapter 4, it is possible that LL-37 was produced but remained in storage. Although a deep understanding of the mechanism by which polyplexes act was not an objective of this thesis, our observations indicate that not only the transfection efficiency determines which is the ideal vector, but also the processes that continue after the expression of the gene of interest and the type of protein it encodes.

In addition to the antimicrobial potential, the expression of LL-37 in HSEs was shown to be cytocompatible toward human keratinocytes and fibroblasts, promoting cell proliferation (chapter 4). This finding was in line with most of the previous literature reports and could be explained due to the association of LL-37 to cell receptors like the fibroblast growth factor receptor (FGFR) and the epidermal growth factor receptor (EGFR).[25] However, a recent study in which the *in vitro* cytocompatibility of LL-37 with stem cell metabolism and chondrogenic differentiation was investigated, reported that concentrations above 25 μg/ml were

toxic and caused cell death in more than 70%.[26] Thus, it is important to determine the optimal concentration of LL-37 in relation to the cell type for future research.

The expression of LL-37 in HSEs also did not affect the formation of the dermo-epidermal compartments, which showed morphological characteristics similar to normal skin. These characteristics together with the antimicrobial properties are promising for the therapeutic use of our skin model since it has advantages compared to the use of antibiotics, due to the natural difficulty that microorganisms have to acquire resistance to the host defense peptides such as LL-37. However, care must be taken since the first bacterial resistance against LL-37 has been developed in *Escherichia coli* strains.[27]

OVERCOMING THE MECHANICAL LIMITATIONS OF HSEs

Fibrin is a biopolymer of interest in tissue engineering for building HSEs that can be used in wound healing. It can be easily obtained from the patient's blood, which makes it a suitable alternative in clinical protocols for autologous use. However, after a deformation process, fibrin loses high amounts of water, which subsequently makes it mechanically unstable and, finally, difficult to manipulate.[28]

Although the mechanical stability of HSEs should not interfere with the secretion of the LL-37 peptide and therefore with their antimicrobial activity, it is a condition that must be improved in order to broaden their clinic applications. The combination of fibrin with other natural polymers such as agarose is considered a novel strategy to improve the overall biomechanical and structural properties of the fibrin while supporting cell functions.[29] The viscoelastic properties and response of human dermal fibroblasts to fibrin-agarose (FA) hydrogels produced with varying concentrations of agarose showed that the addition of agarose increased the stiffness of the FA hydrogels and prevented the contraction of the matrix, which could solve one of the limitations for handling, transport, implantation and performance of these hydrogels[30] (chapter 5). Besides, the viscoelastic properties of FA hydrogels were strongly correlated with the increase in agarose concentration, and in turn, these properties affected cell behavior, mainly cell proliferation and metabolism. Recent studies have also found a potential impact of the hydrogel viscoelasticity on cellular behaviors, such as cell spreading, proliferation, and differentiation.[31] The role of time-dependent mechanics on cell biology remains largely unclear and ripe for

further exploration. The extracellular matrix (ECM) has been shown to be a key regulator of tissue mechanics, which in turn affects various aspects of cell behavior. This has typically been studied in the context of purely elastic matrices and it is unclear how cells interpret these signals in the context of viscoelastic matrices. Cells generate forces and deformations on substrates in a highly dynamic manner.[31] These interactions lead to a complex cellular response that regulates cellular gene expression (mechanotransduction). Therefore, tools and approaches that allow deciphering cell-matrix interactions with greater spatiotemporal resolution are needed, e.g. super-resolution imaging in three dimensions, molecular force sensors, and materials with dynamically tunable mechanical properties, which could address this need providing detailed information on the interactions and dynamic forces that occur between cells and viscoplastic matrices.

Further elucidation of this topic could substantially advance our understanding of cell-matrix interactions and guide the design of improved biomaterials for regenerative medicine,[32] such as HSEs increasingly similar to native skin.

This thesis also demonstrated the importance of interdisciplinary research to propose novel alternatives with a view to improving people's quality of life. The development of HSE with antimicrobial properties was achieved by coupling tissue engineering with disciplines such as gene therapy and biomaterial science. Thanks to the combination of these disciplines, most of the objectives proposed in this work were achieved. However, there are still some questions to be resolved: 1) will HSEs have a similar antimicrobial activity against other bacterial strains? 2) could LL-37 secretion contribute to wound healing?. By solving these questions, the clinical applications of the model could be broadened to prevent infections in skin wounds.

In addition, the HSEs could be evaluated in combination with 1% agarose to improve its mechanical properties and evaluate its effects on cell proliferation and metabolism. Furthermore, since the connection between matrix viscoelasticity and cell signaling could activate transcription factors, which in turn could regulate protein expression,[33] it will be interesting to evaluate the effects of viscoelastic properties of HSEs on LL-37 expression.

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Summary

Non-healing skin wounds are a significant socioeconomic burden due to their high prevalence worldwide. The inefficient recovery of autologous tissue in these wounds increases the susceptibility of patients to infections caused by microorganisms resistant to conventional antimicrobial therapies. These infections increase local tissue damage and delay wound healing, which leads to the development of septic processes that can be life-threatening. Thus, novel approaches are urgently needed to prevent skin wound infections without using antibiotics in order to avoid development of bacterial resistance.

The field of tissue engineering has made significant progress through the application of gene delivery. Gene therapy can be used to enhance existing cellular activities in tissue substitutes. This is achieved by genetically modifying the cells that are grown in the scaffolds or by transferring genes to the site of injury. In skin tissue engineering, epidermal keratinocytes and/or dermal fibroblasts can be genetically modified with viral or non-viral vectors. These cells are then used to produce three-dimensional human skin equivalents (HSEs), which can act as "bioreactors" *in vivo* to produce and secrete for example antimicrobial peptides **(Chapter 1)**. The aim of the research described in this thesis was the development and validation of a fibrin-based human skin equivalent (HSE) with over-expression of the antimicrobial peptide LL-37 as a tool to prevent infections in skin wounds.

Nonviral approaches as gene transfer vehicles have received significant attention because of their safety. In particular, polyfection and nucleofection are two widely used systems for nonviral gene delivery. Their potential has been attributed to the transfection efficiency achieved, which is influenced in turn by the type of cells transfected and by the plasmid that carries the gene of interest. In **Chapter 2**, we determined the efficiency of transfection and the viability of transfected cells by comparing polyplexes and nucleofection in primary human fibroblasts and keratinocytes. Transfections were performed with plasmids containing a gene of interest (human cathelicidin antimicrobial peptide, also called CAMP gene, the precursor for LL-37) and two reporter genes (red or green fluorescent protein) that either included or did not include an internal ribosome entry site (IRES). The efficiency was measured by flow cytometry in terms of the percentage of cells expressing the reporter gene. The viability of transfected cells was also evaluated. It was found that nucleofection was more efficient than using polyplexes for transfecting fibroblasts and keratinocytes. After transfection, the viability of

fibroblasts was high with both systems. In contrast, nucleofection significantly decreased keratinocyte viability. Furthermore, it was observed that both cell types decreased the expression of the reporter gene when the IRES sequence was located upstream, suggesting a negative effect of IRES on the expression of these reporter genes. These findings highlight that the potential of nucleofection and polyplexes systems depend on the efficiency of transfection achieved and on subsequent cell viability, which in turn are influenced by the type of cells transfected and by the plasmid that carries the gene of interest.

Nonviral transfection of skin cells can lead to the construction of more functional skin grafts, for example through the overexpression of antimicrobial peptides that would prevent early contamination and infection with bacteria. In **Chapter 3**, we have modulated the conditions for producing polyethylenimine polyplexes for nonviral transfection of primary human keratinocytes and fibroblasts and the effects on the expression of LL-37 antimicrobial peptide. Linear and branched polyethylenimine polymers in different weight concentrations were varied for evaluating the formation and colloidal characteristics of the polyplexes. The polyethylenimine/pDNA polyplexes with 19 Nitrogen/ Phosphate ratio, are nanometric particles (400 and 250 nm with linear and branched polyethylenimine, respectively) exhibiting positive surface charge (+30 ± 2 mV). Both kinds of polyplexes allowed the expression of a reporter gene and increased the *CAMP* gene expression in transfected keratinocytes and fibroblasts, however, higher cytotoxicity was observed when polyplexes formed with branched polyethylenimine were used. Moreover, cell culture supernatants from transfected cells with linear polyethylenimine/pDNA polyplexes showed enhanced antimicrobial activity (95.8% decrease in bacterial growth) against a *Staphylococcus aureus* strain *in vitro*. The results of the polyethylenimine/pDNA polyplexes formation allowed to develop an improved transfection strategy of skin cells, promoting the production of LL-37 antimicrobial peptide.

After determining the optimal conditions for producing polyethylenimine/pDNA polyplexes, we produced and evaluated Human Skin Equivalents (HSEs) containing transfected primary fibroblasts and keratinocytes to overexpress the antimicrobial peptide LL-37 **(Chapter 4)**. The effect of LL-37 on the metabolic activity of normal HSEs was evaluated before the construction of the transfected HSEs. In addition, the antimicrobial efficacy of LL-37 against *Pseudomonas aeruginosa* and *Staphylococcus aureus* was evaluated. Subsequently, the levels of LL-37 in the culture supernatants of transfected HSEs, as well as the local expression were determined. It was found that LL-37 treatment significantly promoted the cellular proliferation of HSEs. Furthermore, HSEs that expressed elevated levels of LL-37 were shown to possess histological characteristics close to the normal skin and displayed enhanced antimicrobial activity against *S. aureus in vitro*. These findings demonstrate that HSEs expressing LL-37 through nonviral modification of skin cells is a promising approach for the prevention of bacterial colonization in wounds.

While functionally modified HSEs are promising tissue engineering approaches for the prevention of skin wound infections, their mechanical characteristics must be improved to support their future clinical applications. Fibrin has been described as an ideal biomaterial for developing hydrogel scaffolds, due to its physiological role in wound healing and the ability to be remodeled by cells. However, the poor mechanical properties of fibrin-based hydrogels make them much weaker than native tissues, which limits their use in clinical practice. To overcome this limitation, fibrin-agarose (FA) hydrogels have been developed and successfully used for the preparation of different bioengineered tissues. Nevertheless, there is still a need to understand the viscoelastic properties of these FA hydrogels in relation to agarose concentration and its effect on cell behavior and function. In **Chapter 5,** we have characterized the viscoelastic properties of FA hydrogels prepared at different agarose concentrations (0%, 0.5%, 1%, 1.5%, and 2%), and their influence on the biological behavior of seeded human dermal fibroblasts. Cell-seeded and unseeded FA hydrogels were tested in uni-axial compression mode on a low-load compression tester (LLCT). The addition of agarose significantly correlated with mechanical and structural changes in FA hydrogels. The stiffness, relaxation time constants 1(τ1) and 2 (τ2), and fiber diameter increased with addition of agarose, whereas the porosity decreased. The main changes in cell behavior (metabolic activity, and cell proliferation) occurred in

the early stages of culture and were related to the displacement of fast (τ1) and intermediate (τ2) Maxwell elements. Subsequently, fibroblasts seeded in FA hydrogels in concentrations lower than 1% agarose improved their vitality after two weeks of culture, which was demonstrated by the expression of F-actin (actin filaments) and the collagen deposition. Collectively, these results confirmed that cell behavior in FA hydrogels are strongly influenced by their viscoelastic and not the elastic properties, which in turn were coupled with the network structure of the hydrogel. Therefore, viscoelasticity is an important parameter to consider when designing FA hydrogels for tissue engineering application. In a future perspective, the addition of agarose at a concentration of 1% could favor the production of cytocompatible and mechanically stable HSEs.

The HSEs with overexpression of the LL-37 peptide produced in this work constitute a promising alternative for the prevention of skin wound infections, avoiding the use of antibiotics, and offering an alternative to autologous skin grafts. Future research could focus on evaluating the extent of the antimicrobial activity of the model against different bacterial strains. Furthermore, the mechanical properties of HSEs could be improved through the addition of biopolymers such as agarose to broaden their clinical potential **(Chapter 6)**.

Resumen

Las heridas cutáneas que no cicatrizan son una carga socioeconómica importante debido a su alta prevalencia en todo el mundo. La recuperación ineficaz de tejido autólogo en estas heridas aumenta la susceptibilidad de los pacientes a infecciones provocadas por microorganismos resistentes a las terapias antimicrobianas tradicionales. Estas infecciones aumentan el daño tisular local y retrasan la cicatrización de las heridas, lo que conduce al desarrollo de procesos sépticos que pueden poner en peligro la vida. Por lo tanto, se necesitan enfoques novedosos para prevenir infecciones de las heridas en la piel sin usar antibióticos para evitar el desarrollo de resistencia bacteriana.

El campo de la ingeniería de tejidos ha logrado un progreso significativo a través de la aplicación de la entrega de genes. La terapia génica se puede utilizar para mejorar las actividades celulares existentes en los sustitutos de tejidos. Esto se logra modificando genéticamente las células que crecen en los andamios o transfiriendo genes al sitio de la lesión. En la ingeniería de tejidos cutáneos, los queratinocitos epidérmicos y/o los fibroblastos dérmicos pueden modificarse genéticamente con vectores virales o no virales. Estas células se utilizan luego para producir equivalentes tridimensionales de piel humana que pueden actuar como "biorreactores" *in vivo* para producir y secretar, por ejemplo, péptidos antimicrobianos **(Capítulo 1)**. El objetivo de la investigación descrita en esta tesis fue el desarrollo y validación de un equivalente de piel humana (HSE, por sus siglas en inglés) a base de fibrina con sobreexpresión del péptido antimicrobiano LL-37 como herramienta para prevenir infecciones en heridas cutáneas.

Los enfoques no virales como vehículos de transferencia genética han recibido una atención significativa debido a su seguridad. En particular, la polifección y la nucleofección son dos sistemas ampliamente utilizados para la administración de genes no virales. Su potencial se ha atribuido a la eficiencia de transfección alcanzada, que está influenciada a su vez por el tipo de células transfectadas y por el plásmido que porta el gen de interés. En el **Capítulo 2**, determinamos la eficiencia de la transfección y la viabilidad de las células transfectadas comparando poliplexes y nucleofección en fibroblastos y queratinocitos humanos primarios. Las transfecciones se realizaron con plásmidos que contenían un gen de interés (gen del péptido antimicrobiano de la catelicidina humana, también llamado gen CAMP, el precursor de LL-37) y dos genes reporteros (proteína fluorescente roja o verde) que incluían o no un sitio interno de entrada al ribosoma (IRES, por sus siglas en inglés). La eficiencia se midió mediante citometría de flujo en términos del porcentaje de células que expresan el gen reportero.

También se evaluó la viabilidad de las células transfectadas. Se encontró que la nucleofección era más eficiente que el uso de poliplexes para transfectar fibroblastos y queratinocitos. Después de la transfección, la viabilidad de los fibroblastos fue alta con ambos sistemas. Por el contrario, la nucleofección disminuyó significativamente la viabilidad de los queratinocitos. Además, se observó que ambos tipos de células disminuían la expresión del gen reportero cuando la secuencia de IRES se localizaba corriente arriba, lo que sugiere un efecto negativo de IRES sobre la expresión de estos genes reporteros. Estos hallazgos destacan que el potencial de los sistemas de nucleofección y poliplexes depende de la eficiencia de transfección lograda y de la viabilidad celular posterior, que a su vez están influenciadas por el tipo de células transfectadas y por el plásmido que porta el gen de interés.

La transfección no viral de células de la piel puede conducir a la construcción de injertos de piel más funcionales, por ejemplo, a través de la sobreexpresión de péptidos antimicrobianos que evitarían la contaminación y la infección temprana con bacterias. En el **Capítulo 3**, hemos modulado las condiciones para producir poliplexes de polietilenimina para la transfección no viral de queratinocitos y fibroblastos humanos primarios, y evaluado sus efectos sobre la expresión del péptido antimicrobiano LL-37. Se prepararon polímeros de polietilenimina lineales y ramificados en diferentes concentraciones de peso para evaluar la formación y las características coloidales de los poliplexes. Los poliplexes de polietilenimina /pDNA con una relación Nitrógeno/Fosfato de 19, son partículas nanométricas (entre 400 y 250 nm con polietilenimina lineal y ramificada, respectivamente) que exhiben carga superficial positiva (+30 ± 2 mV). Ambos tipos de poliplexes permitieron la expresión del gen reportero y aumentaron la expresión del gen *CAMP* en queratinocitos y fibroblastos transfectados; sin embargo, se observó una mayor citotoxicidad cuando se utilizaron poliplexes formados con polietilenimina ramificada. Además, los sobrenadantes de cultivos celulares de las células transfectadas con poliplexes de polietilenimina lineal mostraron una actividad antimicrobiana mejorada (disminución del 95,8% en el crecimiento bacteriano) contra una cepa de *Staphylococcus aureus in vitro*. Los resultados de la formación de los poliplexes de polietilenimina permitieron desarrollar una estrategia de transfección mejorada de las células de la piel, promoviendo la producción del péptido antimicrobiano LL-37.

Después de determinar las condiciones óptimas para producir poliplexes de polietilenimina, producimos y evaluamos equivalentes de piel humana (HSEs) que contienen fibroblastos y queratinocitos primarios transfectados para sobreexpresar el

péptido antimicrobiano LL-37 **(Capítulo 4)**. El efecto del LL-37 sobre la actividad metabólica de los HSEs normales se evaluó antes de la construcción de los HSEs transfectados. Además, se evaluó la eficacia antimicrobiana del LL-37 contra *Pseudomonas aeruginosa* y *Staphylococcus aureus*. Posteriormente, se determinaron los niveles de LL-37 en los sobrenadantes de cultivo de HSEs transfectados, así como la expresión local. Se encontró que el tratamiento con LL-37 promovió significativamente la proliferación celular de los HSEs. Además, se demostró que los HSEs que expresaban niveles elevados de LL-37 poseían características histológicas cercanas a las de la piel normal y mostraban una actividad antimicrobiana mejorada contra *S. aureus in vitro*. Estos hallazgos demuestran que los HSEs que expresan LL-37 a través de la modificación no viral de las células de la piel son un enfoque prometedor para la prevención de la colonización bacteriana en las heridas de piel.

Si bien los HSEs funcionalmente modificados constituyen enfoques prometedores de ingeniería de tejidos para la prevención de infecciones de heridas en la piel, sus características mecánicas deben mejorarse para respaldar sus futuras aplicaciones clínicas. La fibrina se ha descrito como un biomaterial ideal para desarrollar hidrogeles, debido a su papel fisiológico en la cicatrización de heridas y la capacidad de ser remodelada por las células. Sin embargo, las propiedades mecánicas pobres de los hidrogeles a base de fibrina los hacen mucho más débiles que los tejidos nativos, lo que limita su uso en la práctica clínica. Para superar esta limitación, se han desarrollado y utilizado con éxito hidrogeles de fibrina-agarosa (FA) para la preparación de diferentes tejidos. Sin embargo, todavía existe la necesidad de comprender las propiedades viscoelásticas de estos hidrogeles FA en relación con la concentración de agarosa y su efecto sobre el comportamiento y la función celular. En el **Capítulo 5**, hemos caracterizado las propiedades viscoelásticas de los hidrogeles FA preparados en diferentes concentraciones de agarosa (0%, 0,5%, 1%, 1,5% y 2%) y su influencia en el comportamiento biológico de los fibroblastos dérmicos humanos sembrados. Los hidrogeles de FA con células y sin células se probaron en modo de compresión uniaxial en un probador de compresión de baja carga (LLCT, por sus siglas en inglés). La adición de agarosa se correlacionó significativamente con los cambios mecánicos y estructurales en los hidrogeles FA. La rigidez, las constantes de tiempo de relajación 1 ($\tau 1$) y 2 ($\tau 2$) y el diámetro de las fibras aumentaron con la adición de agarosa, mientras que la porosidad disminuyó. Los principales cambios en el comportamiento celular (actividad metabólica y proliferación celular) ocurrieron en las primeras etapas del cultivo y se relacionaron con el desplazamiento de elementos Maxwell rápidos ($\tau 1$) e intermedios ($\tau 2$). Posteriormente, los fibroblastos sembrados en hidrogeles FA en concentraciones

inferiores al 1% de agarosa mejoraron su vitalidad después de dos semanas de cultivo, lo que se demostró por la expresión de los filamentos de actina y la producción de colágeno. En conjunto, estos resultados confirmaron que el comportamiento celular en los hidrogeles FA está fuertemente influenciado por sus propiedades viscoelásticas y no elásticas, y que, a su vez estas propiedades estaban acopladas con la estructura de la red del hidrogel. Por lo tanto, la viscoelasticidad es un parámetro importante por considerar al diseñar hidrogeles FA para aplicaciones de ingeniería de tejidos. En una perspectiva futura, la adición de agarosa a una concentración del 1% podría favorecer la producción de HSEs citocompatibles y más estables mecánicamente.

Los HSEs con sobreexpresión del péptido LL-37 producidos en este trabajo constituyen una alternativa prometedora para la prevención de infecciones de las heridas en la piel, evitando el uso de antibióticos y ofreciendo una alternativa a los injertos cutáneos autólogos. La investigación futura podría centrarse en evaluar el alcance de la actividad antimicrobiana del modelo contra diferentes cepas bacterianas. Además, las propiedades mecánicas de los HSEs podrían mejorarse mediante la adición de biopolímeros como la agarosa para ampliar su potencial clínico **(Capítulo 6).**

Samenvatting

Niet-genezende huidwonden vormen een aanzienlijke sociaal-economische last vanwege hun hoge prevalentie wereldwijd. Het inefficiënte herstel van autoloog weefsel in deze wonden verhoogt de gevoeligheid van patiënten voor infecties veroorzaakt door micro-organismen die resistent zijn tegen conventionele antimicrobiële therapieën. Deze infecties verhogen lokale weefselbeschadiging en vertragen de wondgenezing, wat leidt tot de ontwikkeling van septische processen die levensbedreigend kunnen zijn. Er zijn dus dringend nieuwe benaderingen nodig om huidwondinfecties te voorkomen zonder gebruik van antibiotica om zo de ontwikkeling van bacteriële resistentie te voorkomen.

Op het gebied van tissue engineering is aanzienlijke vooruitgang geboekt door de toepassing van gentherapie. Deze techniek kan worden gebruikt om bestaande cellulaire activiteit in weefselvervangers te verbeteren. Dit wordt bereikt door de cellen die in de zogenoemde “scaffolds” worden gekweekt genetisch te modificeren of door genen naar de plaats van de verwonding over te brengen. Bij huidweefselmanipulatie kunnen epidermale keratinocyten en/of dermale fibroblasten genetisch worden gemodificeerd met virale of niet-virale vectoren. Deze cellen worden vervolgens gebruikt om driedimensionale menselijke huidequivalenten (HHE's) te produceren, die in vivo kunnen fungeren als "bioreactoren" om bijvoorbeeld antimicrobiële peptiden te produceren en uit te scheiden **(Hoofdstuk 1)**. Het doel van het onderzoek beschreven in dit proefschrift was de ontwikkeling en validatie van een op fibrine gebaseerd humaan huidequivalent (HHE) met overexpressie van het antimicrobiële peptide LL-37 als middel om infecties in huidwonden te voorkomen.

Niet-virale benaderingen als vehikels voor genoverdracht hebben veel aandacht gekregen vanwege hun veiligheid. In het bijzonder zijn polyfectie en nucleofectie twee veelgebruikte systemen voor niet-virale genafgifte in ontvangende cellen. Hun potentieel wordt toegeschreven aan de bereikte transfectie-efficiëntie, die op zijn beurt wordt beïnvloed door het type getransfecteerde cellen en door het plasmide dat het betreffende gen draagt. In **Hoofdstuk 2** hebben we de efficiëntie van transfectie en de levensvatbaarheid van getransfecteerde cellen bepaald door het gebruik van polyplexen en nucleofectie in primaire humane fibroblasten en keratinocyten te vergelijken. Transfecties werden uitgevoerd met plasmiden die het CAMP gen bevatten, dat staat voor humaan cathelicidin antimicrobieel peptide, hetgeen de voorloper van LL-37 is, en verder twee reportergenen (rood of groen

fluorescerend eiwit) die al dan niet een interne ribosoomingang (IRES) bevatten. De efficiëntie werd gemeten met flowcytometrie in termen van het percentage cellen dat het reportergen tot expressie brengt. Verder was nucleofectie efficiënter dan het gebruik van polyplexen voor het transfecteren van fibroblasten met betrekking tot hun levensvatbaarheid, terwijl er geen significante verschillen werden gevonden tussen beide systemen van transfectie wanneer ze werden toegepast op keratinocyten. Na transfectie was de levensvatbaarheid van fibroblasten bij beide systemen sowieso hoog. Daarentegen verminderde nucleofectie de levensvatbaarheid van keratinocyten aanzienlijk. Verder werd waargenomen dat beide celtypes de expressie van het reportergen verminderden wanneer de IRES-sequentie stroomopwaarts was gelokaliseerd, wat een negatief effect van IRES op de expressie van deze reportergenen suggereert. Deze bevindingen benadrukken dat het potentieel van nucleofectie- en polyplexsystemen afhangt van de efficiëntie van de transfectie die wordt bereikt en van de daaropvolgende levensvatbaarheid van de cellen, die op hun beurt worden beïnvloed door het type cellen dat wordt getransfecteerd en door het plasmide dat het betreffende gen draagt.

Niet-virale transfectie van huidcellen kan leiden tot de constructie van meer functionele huidtransplantaten, bijvoorbeeld door de overexpressie van antimicrobiële peptiden die vroege besmetting en infectie met bacteriën zouden kunnen voorkomen. In **Hoofdstuk 3** hebben we de condities gemoduleerd voor het produceren van polyethyleenimine polyplexen voor niet-virale transfectie van primaire humane keratinocyten en fibroblasten en de effecten ervan op de expressie van het LL-37 antimicrobieel peptide. Lineaire en vertakte polyethyleenimine polymeren in verschillende gewichtsconcentraties werden gekozen voor het evalueren van de vorming en colloïdale eigenschappen van de polyplexen. De polyethyleenimine/pDNA-polyplexen met een stikstof/fosfaatverhouding van 19 zijn nanometrische deeltjes (400 en 250 nm voor lineair en vertakt polyethyleenimine, respectievelijk) met een positieve oppervlaktelading (+30 2 mV). Beide soorten polyplexen maakten de expressie van een reportergen mogelijk en verhoogden de CAMP-genexpressie in getransfecteerde keratinocyten en fibroblasten. Er werd echter een hogere cytotoxiciteit waargenomen wanneer polyplexen gevormd met vertakte PEI werden gebruikt. Celkweeksupernatanten van getransfecteerde cellen met lineaire polyethyleenimine/pDNA-polyplexen vertoonden verhoogde antimicrobiële activiteit zoals gemeten door de afname van bacteriegroei (95,8 %) van *Staphylococcus aureus* bacteriën in vitro. De studie van de

vorming van polyethyleenimine/pDNA-polyplexen maakte het mogelijk om een verbeterde transfectiestrategie van huidcellen te ontwikkelen, waardoor de productie van LL-37 antimicrobieel peptide werd bevorderd.

Na het bepalen van de optimale omstandigheden voor het produceren van polyethyleenimine/pDNA-polyplexen, produceerden en evalueerden we menselijke huidequivalenten die getransfecteerde primaire fibroblasten en keratinocyten bevatten om het antimicrobiële peptide LL-37 tot overexpressie te brengen **(Hoofdstuk 4)**. Het effect van LL-37 op de metabole activiteit van de cellen in normale HHE's werd geëvalueerd vóór de constructie van de getransfecteerde HHE's, evenals de antimicrobiële werkzaamheid tegen de bacteriën *Pseudomonas aeruginosa* en *Staphylococcus aureus*. Vervolgens werden de niveaus van LL-37 in de kweeksupernatanten van getransfecteerde HHE's bepaald, evenals de lokale expressie. Er werd gevonden dat behandeling met het LL-37 voorloper gen de cellulaire proliferatie in HHE's significant bevorderde. Bovendien werd aangetoond dat HHE's die verhoogde niveaus van het eiwit LL-37 tot expressie brengen, histologische kenmerken hebben die dicht bij die van de normale huid liggen, en tevens een verhoogde antimicrobiële activiteit vertonen tegen *Staphylococcus aureus* in vitro. Deze bevindingen tonen aan dat HHE's die LL-37 verhoogd tot expressie brengen door niet-virale modificatie van huidcellen een veelbelovende benadering kan zijn voor de preventie van bacteriële kolonisatie in wonden.

Hoewel functioneel gemodificeerde HHE's veelbelovende benaderingen voor huidweefselmanipulatie zijn ten behoeve van infectiepreventie, moeten hun mechanische eigenschappen worden verbeterd om hun toekomstige klinische toepassingen te ondersteunen. Fibrine is beschreven als een ideaal biomateriaal voor het ontwikkelen van hydrogel scaffolds, vanwege de fysiologische rol bij wondgenezing en het vermogen om door cellen te worden hermodelleerd. De slechte mechanische eigenschappen van op fibrine gebaseerde hydrogels maken ze echter veel zwakker dan de natuurlijke weefsels, wat hun gebruik in de klinische praktijk beperkt. Fibrine-agarose (FA) hydrogels zijn ontwikkeld om de mechanische sterkte te verbeteren en worden met succes gebruikt voor de bereiding van verschillende gebioengineerde weefsels. Desalniettemin is er nog steeds behoefte om de visco-elastische eigenschappen van deze FA-hydrogels te begrijpen in relatie tot de agarose concentratie en het effect ervan op celgedrag en -functie. In **Hoofdstuk 5** karakteriseren we deze visco-elastische eigenschappen bij verschillende

agaroseconcentraties (0%, 0,5%, 1%, 1,5% en 2%) en hun invloed op het biologische gedrag van gezaaide menselijke huidfibroblasten. De FA-hydrogels werden getest in een uni-axiale compressiemodus met een compressietester met lage belasting (LLCT). De toevoeging van agarose correleerde significant met mechanische en structurele veranderingen in de FA-hydrogels. De stijfheid, relaxatietijdconstanten 1 (τ1) en 2 (τ2), en vezeldiameter namen toe met toevoeging van agarose, terwijl de porositeit afnam. De belangrijkste veranderingen in celgedrag (metabole activiteit en celproliferatie) deden zich voor in de vroege stadia van de kweek en waren gerelateerd aan de verplaatsing van de snelle (τ1) en intermediaire (τ2) Maxwell-elementen. Fibroblasten gezaaid in de hydrogels met agarose concentraties lager dan 1% verhoogden hun vitaliteit na twee weken kweken, hetgeen werd aangetoond door de expressie van actinefilamenten en de collageenafzetting. Gezamenlijk bevestigen deze resultaten dat celgedrag in FA-hydrogels sterk wordt beïnvloed door hun visco-elastische en niet door de elastische eigenschappen, welke op hun beurt gekoppeld zijn aan de netwerkstructuur van de hydrogel. Daarom is visco-elasticiteit een belangrijke parameter om te overwegen bij het ontwerpen van FA-hydrogels voor weefseltechnologie toepassing. In een toekomstperspectief zou de toevoeging van agarose in een concentratie van 1% de productie van cytocompatibele en mechanisch stabiele HHE's kunnen bevorderen.

De HHE's met overexpressie van het LL-37-peptide beschreven in dit proefschrift vormen een veelbelovende benadering voor de preventie van huidwondinfecties, zonder het gebruik van antibiotica, en een alternatief voor autologe huidtransplantaten. Toekomstig onderzoek zou zich moeten richten op het evalueren van de mate van antimicrobiële activiteit tegen verschillende andere bacteriestammen. Bovendien kunnen de mechanische eigenschappen van HHE's verder worden geoptimaliseerd door de toevoeging van biopolymeren zoals agarose om daarmee hun klinische potentieel te vergroten **(Hoofdstuk 6)**.

Acknowledgments

Although I find it hard to believe, this PhD journey has come to an end. Looking back over the last few years, I am grateful for every decision I made, for every success, and mistake, that brought me to this point and that made me grow on an academic and personal level. There were many lessons learned and I believe, that one of the most important ones was that there is no unattainable accomplishment when you have the support of others. This thesis would not have been possible without the support of many of you and for that I am incredibly grateful.

Dear **Prashant** and **Theo**, thank you very much for allowing me to do my PhD in the BME group. This has been the most wonderful and enriching experience I have had in my life. You were the best team to bring this multidisciplinary research to a happy ending. **Prashant,** I am sincerely grateful for your dedication and support over the past three years. Thank you for the respect you always showed, for listening to my suggestions and ideas, and for the direction and accuracy you provided to them. Thank you for encouraging me and for being willing to listen even in my difficult moments. Your words always motivated me to not give up. **Theo,** your door was always open for me. Thank you for the patience with which you answered my questions, for sharing your expertise, and for guiding me through the design and analysis of my experiments. I will never forget when I met you in Colombia. I had to present my project to you, and it went horribly, because I was very nervous!. But in the end, you smiled and only highlighted the positive. That smile on your face and your optimism always gave me a dose of reassurance.

Dear **Henny**, thank you very much for your support and all your contributions to this work. Your ability to guide my ideas has been an invaluable contribution, not only in the development of this thesis but also in my training as a researcher. Thank you for the thoroughness with which you always reviewed my manuscripts and, above all, for your availability and patience that made our discussions always fruitful. Your attention to detail on research always impressed me! You have my great admiration and respect.

Profe Luz Marina (Mona), no existen palabras!. Unos cuantos renglones nunca serán suficientes para expresar la inmensa gratitud que siento. Sin tu apoyo y confianza nada de esto hubiera sido posible. Infinitas gracias por creer en mi incluso mucho antes de que yo misma lo hiciera. Por compartir tu pasión por la ciencia y por ser una fuente de inspiración para quienes te rodean. Gracias a ti se me abrieron las

puertas a una cantidad de experiencias profesionales y personales que me han hecho crecer en todos los aspectos de mi vida. Siempre te llevaré en mi corazón.

Naty, tu experiencia y valiosos aportes fueron indispensables en el desarrollo de esta tesis. Gracias por el apoyo y la confianza que depositaste en mí. Por la paciencia y la dedicación con la que me guiaste durante los primeros años de doctorado, y por haber estado dispuesta a ayudarme siempre que lo necesité.

Un agradecimiento especial a los miembros de mi comité tutorial, **Dr. Claudia Bernal**, **Dr. Juan Carlos Cruz** y **Dr. Mónica Mesa**, por el tiempo dedicado a mi proyecto de investigación y por todos los valiosos consejos y sugerencias.

I would like to express my deepest gratitude to **Prof. Marco Harmsen, Prof. Paul Jutte, Prof. Erik Biessen, and Prof. María Del Pilar Jiménez** for agreeing to be part of the reading committee and for the valuable time dedicated to evaluating my thesis.

This work would not have been possible without the financial support of **COLCIENCIAS National Doctoral program (code 727-2015)** and the **Abel Tasman Talent Program (ATTP)** of the University of Groningen, to whom I am sincerely grateful.

Special thanks to the entire scientific staff: **Patrick, Brandon, Inge, Jelmer,** and **Romana** for the constructive discussions during the lunch meetings and the Kolff-days.

To the technical staff: **Reinier, Gesinda, Willem, Ed, Corien, Jelly, Marja, Betsy, Joop, Hans** and **Willy**. Thank you for the patience and dedication with which you train the students! Your experience is invaluable, and I am very grateful for the kindness with which you were always willing to help me. **Ina** and **Wya**, thank you for your support in all administrative matters, for your efficiency and good disposition.

Doing a PhD in two countries has resulted in a large number of colleagues throughout the years. It is impossible to mention everyone, but I am deeply grateful for all the good memories.

To my colleagues and friends at the GITTC: **Yina, Paulina, Carolina, Gabriel, Sergio, Ricardo** y **Diana**. Gracias por todos los momentos compartidos, por nuestras discusiones durante los clubes de revista, los almuerzos, las mañanas de café con pan de queso, las risas, las lágrimas y el apoyo incondicional. ¡No se imaginan cuánto los extrañé!

To all my colleagues and friends at BME: **Mari, Laís, Thaís, Damla, Abby, Valentina, Sara (Lu), Torben, Neda, Claudia, Can, Jeroen, Hongping, Ke, Fenghua, Linyan, Yue, Roksana, Arturo**. Thanks for the scientific discussions, the coffees, the lunches, and the conversations in the corridors. I wish you all the success in your careers! To my office mates: **Olga S** and **Thamir,** thank you for cheering me up and making my last days of writing more bearable.

Dear **Yanyan,** I am very lucky to have met you in Groningen. Thank you for your sincere friendship, for always being willing to listen to me and giving me advice when I most needed it. Thank you for our coffee afternoons, the walks around the city, and for our trips together. I appreciate you very much!

To my friends **Olga C** and **Carlos**, I will never forget all the support you gave me during my stay in the Netherlands. People like you make the world a better place! **Viraj** and **Yoshita**, meeting you was a wonderful gift, thank you for your advice, for all the delicious dinners and for opening the doors of your home to me.

A special thanks to my paranimfen, whom I deeply appreciate and admire:

Alejo, quiero agradecerte porque desde que te conocí siempre he podido contar contigo. Gracias por tus consejos y por darme ánimos en los momentos difíciles. Tenerte como colega ha sido un privilegio y como amigo un gran regalo. La determinación con la que siempre consigues lo que te propones siempre me ha sorprendido, y estoy segura de que te esperan muchos éxitos en tu vida profesional. Sigue brillando!.

Cata, soy muy afortunada de tenerte como amiga. Siempre estuviste ahí desde el comienzo de esta aventura apoyándome incondicionalmente. A pesar de estar tan lejos de nuestras familias y amigos, nunca me sentí sola gracias a ti. Eres una mujer increíble, inteligente y centrada (así pienses que no es así). No te imaginas lo mucho que te admiro por tu dedicación y la tranquilidad con la que trabajas por alcanzar tus metas.

Anita, gracias por tu apoyo incondicional y por cada palabra de motivación. Cata y tú son dos de mis personas favoritas en el mundo y sé que no importa dónde estemos, siempre seremos amigas.

To the friends that the Netherlands brought me:

Juanito, Fabio, Eli, Angie, Javi and **Marthijs.** Thank you very much for everything we shared, the best memories of my life in Groningen have you in them and I hope to have the opportunity to meet you again in any other place in the world. **Francisco**, I want to thank you especially not only for your support and your sincere friendship, but also for your invaluable contribution in the last chapter of this thesis. ¡Los quiero mucho chicos!

To my housemates, **Kieu, Natalia, Pata** and **Eva**, thank you for making our cozy house a home. For the conversations, dinners, parties, and evenings of baking cakes. I will miss you so much!

A mi familia, en especial a aquellos que siempre han estado presentes a pesar de la distancia. Gracias por su amor y apoyo incondicional.

Mami, tu amor es el motor de mi vida y todo lo que soy es gracias a ti. Estar lejos de casa ha sido un sacrificio enorme, pero tú nunca dejaste que me diera por vencida. Gracias por darme las fuerzas suficientes para perseguir mis sueños. Te amo mucho y mi mayor recompensa siempre serás tú.

Tío **Rodrigo**, nunca olvidaré todo lo que has hecho por mí. Gracias por apoyarme en todas las etapas de mi vida y por enseñarme con tu ejemplo que todo es posible si te lo propones. Esta tesis también es para ti.

Maria Isabel Patiño Vargas

November 2021

Curriculum Vitae

Maria Isabel was born on the 18th of April 1991 in Medellin, Colombia. She completed a five-year B.Sc. degree in Microbiology and Bioanalysis at the University of Antioquia, in Medellin. During her studies, she joined as a Young Researcher in the Tissue Engineering and Cell Therapy Research Group within the Faculty of Medicine. There she carried out her degree project focused on the production of decellularized tracheal extracellular matrices for the repair of the airway. This project obtained a mention of recognition by the School of Microbiology.

In 2016, Maria Isabel started her doctoral program at the Corporation of Basic Biomedical Sciences of the University of Antioquia with an emphasis on Tissue Engineering and Regenerative Medicine. She was awarded a scholarship (COLCIENCIAS national doctorates) to finance her project. Then she moved to Groningen, the Netherlands to start a double degree program at the University of Groningen, in the department of Biomedical Engineering. During that period, she worked under the supervision of Dr. Prashant Sharma, Dr. Theo van Kooten, and Prof. Henny C. van der Mei. Her research focused on the development and validation of a robust fibrin-based human skin equivalent with over-expression of the antimicrobial peptide LL-37 to prevent infections in skin wounds. During her PhD she participated in different scientific events, including the TERMIS World Congress, the European Pharma Congress, and the European Society for Artificial Organs Congress. Maria is ready to accept new challenges that will allow her to enrich her career in research.