UNIVERSIDADE DA BEIRA INTERIOR
Ciências da Saúde

Development of new biomaterials to be applied as Skin Substitutes

Maximiano José Prata Ribeiro

Tese para obtenção do Grau de Doutor em
Biomedicina
(3° ciclo de estudos)

Orientador: Prof. Doutor Ilídio Joaquim Sobreira Correia
Coorientadora: Prof. Doutora Paula Isabel Teixeira Gonçalves Coutinho Borges

Covilhã, outubro de 2014

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Dedicatory

To my family, my supervisors and my friends

“Imagination is more important than knowledge. For knowledge is limited to all we now know and understand, while imagination embraces the entire world, and all there ever will be to know and understand.”

Albert Einstein

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First of all I would like to thank my supervisors, Professor Doutor Ilídio Correia and Professora Doutora Paula Coutinho for their precious scientific support, promptness, care and friendship. I am also grateful to them listened my ideas and have always been patient and encouraging me when the difficulties appeared. It has been a huge privilege for me to work and learn with them.

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Moreover, I would like to express my gratitude to all the persons that directly contributed for this work, with a special appreciation to my group colleagues.

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List of papers

Publications in Peer-Reviewed International Scientific Journals included in the thesis

I. Development of a new chitosan hydrogel for wound dressing
M.P. Ribeiro, A. Espiga, D. Silva, P. Baptista, J. Henriques, C. Ferreira, J.C. Silva, J.P. Borges, E. Pires, P. Chaves, I.J. Correia
Wound Repair and Regeneration. 2009 (17) 817-824.
DOI: 10.1111/j.1524-475X.2009.00538.x

II. Dextran based-hydrogel containing chitosan microparticles loaded with growth factors to be used in wound healing
M.P. Ribeiro, P.I. Morgado, S.P. Miguel, P. Coutinho, I.J. Correia
Materials Science and Engineering: C. 2013 (33) 2958-2966.
DOI: 10.1016/j.msec.2013.03.025

III. Thermoresponsive chitosan-agarose hydrogel for skin regeneration
S.P. Miguel, **M.P. Ribeiro**, H. Brancal, P. Coutinho, I.J. Correia
Carbohydrate Polymers. 2014 (111) 366-373.
DOI: 10.1016/j.carbpol.2014.04.093

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A. Palmeira-de-Oliveira, **M.P. Ribeiro**, R. Palmeira-de-Oliveira, C. Gaspar, S. Costa-de-Oliveira, I.J. Correia, J. Martinez-de-Oliveira, J. Queiroz, A. Rodrigues
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DOI:10.1159/000314023

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DOI: 10.1002/anie.201200362

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Tissue Engineering Part C: Methods. 2014,*in press*
DOI: 10.1089/ten.tec.2013.0738.

VI. Poly(vinyl alcohol)/chitosan asymmetric membranes: highly controlled morphology toward the ideal wound dressing
P.I. Morgado, P.F. Lisboa, **M.P. Ribeiro**, S.P. Miguel, P.C. Simões, I.J. Correia, A. Aguiar-Ricardo
Journal of Membrane Science. 2014 (469) 262-271.
DOI: 10.1016/j.memsci.2014.06.035

List of scientific communications

Invited Lectures presented during the PhD

I. **M.P. Ribeiro,** S.P. Miguel P. Coutinho, I.J. Correia. "Biomateriais na regeneração da pele" I Ciclo de Conferências "Biomédicas à tarde", Covilhã, Portugal, May 7th 2013.

II. **M.P. Ribeiro,** S.P. Miguel P. Coutinho, I.J. Correia. Desenvolvimento de novos biomateriais para regeneração da pele" II Jornadas de Bioengenharia, Covilhã, Portugal, May 16th 2013.

III. **M.P. Ribeiro,** S.P. Miguel P. Coutinho, I.J. Correia. "Biomateriais na regeneração da pele" I Encontro Nacional de Estudantes de Biotecnologia, Covilhã, Portugal, May 25th 2013.

Oral scientific communications presented during the PhD

I. **M.P. Ribeiro**, P. Coutinho, P.I. Morgado, C. Gaspar, I.J. Correia. "Desenvolvimento de Biomateriais para a Regeneração da Pele". III Jornadas sobre Tecnologia e Saúde, Guarda, Portugal, April 30th, 2010.

II. **M.P. Ribeiro,** P. Coutinho, I.J. Correia. "Desenvolvimento de Hidrogéis para a Regeneração da Pele". *Simposium* 2011 APTFeridas Exponor, Matosinhos, Portugal, November 18th, 2011.

III. **M.P. Ribeiro,** P.I. Morgado, S.P. Miguel, P. Coutinho, I.J. Correia. "Dextran-based hydrogel containing chitosan microparticles loaded with growth factors to be used in wound healing". VI Jornadas sobre Tecnologia e Saúde, Guarda, Portugal, May 3rd, 2013.

IV. S.P. Miguel, **M.P. Ribeiro,** H. Brancal, P. Coutinho, I.J. Correia. "Thermoresponsive chitosan-agarose hydrogel for skin regeneration". IX Annual CICS Symposium, Centro de Investigação em Ciências da Saúde, Universidade da Beira Interior, Covilhã, Portugal, June 30th and July 1st, 2014.

Poster scientific communications presented during the PhD

I. R.B. Restani, P.I. Morgado, **M.P. Ribeiro**, I.J. Correia, A. Aguiar-Ricardo, V.D. Bonifácio. "Smart Dendrimers". 4th International Symposium on Advances in Synthetic and Medical Chemistry, St. Petersburg, Russia, August 21st-25th, 2011.

II. P.I. Morgado, **M.P. Ribeiro**, S.P. Miguel, I.J. Correia, A. Aguiar-Ricardo. "Development of poly(vinyl alcohol) and chitosan wound dressings using supercritical fluids technology". 11º Encontro Nacional de Química-Física, Faculdade de Ciências, Universidade do Porto, Portugal, May 9th-10th, 2013

III. S.P. Miguel, **M.P. Ribeiro,** H. Brancal, P. Coutinho, I.J. Correia. "Thermoresponsive chitosan-agarose hydrogel for skin regeneration". Encontro Bienal das Divisões Técnicas da Sociedade Portuguesa dos Materiais, Universidade da Beira Interior, Covilhã, Portugal, May 21st, 2014.

IV. P.I. Morgado, F. Lisboa, **M.P. Ribeiro**, S.P. Miguel, P.C. Simões, I.J. Correia, A. Aguiar-Ricardo. "scCO_2-phase inversion technique: a sustainable method to produce asymmetric membranes with highly controlled morphology toward the ideal wound dressing". Gordon Research Conference / Seminar on Green Chemistry, The Chinese University of Hong Kong, July 26th-27th, 2014.

Resumo alargado

A pele é o maior órgão do corpo humano e protege/defende os órgãos subjacentes de agentes patogénicos ou outros agentes nocivos. Devido à sua localização, estrutura e funções, a pele, está sujeita a agentes térmicos, mecânicos e químicos potencialmente nocivos que lhe podem causar diferentes tipos de lesões. Estas têm uma grande incidência na população mundial e o seu tratamento implica elevados custos associados.

Após uma lesão, a integridade da pele fica comprometida obrigando o organismo a desencadear o mecanismo de cicatrização para restabelecer a estrutura e funções deste órgão. Este processo é extremamente dinâmico e biologicamente interativo, envolvendo interações entre moléculas da matriz extracelular, mediadores solúveis e várias células (fibroblastos, queratinócitos e leucócitos), que permitem o restabelecimento da integridade ou a substituição do tecido lesado.

De forma a melhorar as hipóteses de sobrevivência dos pacientes e a minimizar a perda de funções vitais da pele, esta tem de ser revestida imediatamente após lesão. Tal necessidade destaca a importância do desenvolvimento de novos revestimentos de pele que melhorem o processo de cicatrização, tornando o menos doloroso, e que simultaneamente, contribuam para o restabelecimento das propriedades nativas da pele num curto período de tempo.

Nos últimos anos, têm vindo a ser desenvolvidos diversos substitutos de pele, utilizando materiais naturais ou sintéticos, sendo alguns deles já utilizados em meio clínico. Contudo, até ao momento nenhum deles foi capaz de restabelecer todas as propriedades da pele e as suas estruturas acessórias.

Tendo isso em conta, o presente estudo teve como principal objetivo o desenvolvimento e caracterização de substitutos de pele para o tratamento de lesões da pele. Para este fim foram usados polímeros naturais, nomeadamente o quitosano, o dextrano e a agarose.

Numa primeira fase do estudo, avaliou-se a aplicabilidade de um hidrogel à base de quitosano para o revestimento de feridas. O quitosano é considerado um biomaterial versátil com propriedades (elevada biocompatibilidade, biodegradabilidade, hemostático, bactericida e baixa antigenicidade) que permitem a sua utilização em aplicações biomédicas. Inicialmente procedeu-se à caraterização morfológica do hidrogel desenvolvido, através de microscopia eletrónica de varrimento. Posteriormente, avaliou-se o perfil citotóxico e de degradação do hidrogel através de ensaios *in vitro*. Por último, foram também realizados ensaios *in vivo* para avaliar o potencial do hidrogel de quitosano no tratamento de queimaduras. Os resultados obtidos revelaram que o hidrogel desenvolvido possui as propriedades necessárias para a aplicação biomédica pretendida, uma vez que os ensaios *in vitro* e *in vivo* revelaram que o hidrogel de quitosano e os seus subprodutos de degradação são biocompatíveis e que o mesmo possui capacidade de promover o processo de cicatrização de feridas cutâneas.

Posteriormente, foi desenvolvido um hidrogel de dextrano com micropartículas de quitosano carregadas com fatores de crescimento (fator de crescimento vascular endotelial e fator de

crescimento epitelial). O dextrano é um polissacárido natural, biocompatível, biodegradável e com elevada versatilidade o que permite a sua utilização na síntese de diferentes hidrogéis. A morfologia do hidrogel produzido foi caracterizada através de microscopia eletrónica de varrimento. Os estudos *in vitro* permitiram avaliar o perfil de toxicidade e degradação do hidrogel. Subsequentemente ratos Wistar foram usados para caracterizar a aplicabilidade do sistema no tratamento de queimaduras na pele. Os resultados obtidos demonstraram que o hidrogel de dextrano pode ser utilizado como um revestimento de pele e como um veículo para transportar fatores de crescimento, de forma a permitir uma mais rápida cicatrização da ferida, sem que tenham sido observados sinais de resposta inflamatória local ou sistémica. Por outro lado, verificou-se que uma aplicação semanal deste substituto da pele carregado com fatores de crescimento contribuiu para reduzir a área da ferida mais rapidamente do que quando os fatores de crescimento são aplicados na forma livre sobre a ferida, a cada dois dias. Além disso, o hidrogel de dextrano pode ser uma excelente opção para a aplicação em feridas com extremidades irregulares, uma vez que o hidrogel é reticulado *in situ* e este pode adaptar-se à forma da ferida.

Num terceiro estudo, foi desenvolvido um hidrogel termoresponsivo constituído por quitosano e agarose e a sua aplicabilidade no processo de cicatrização de feridas foi caracterizada. A agarose é um polissacárido biocompatível extraído a partir de algas marinhas, com capacidade de produzir géis termoresponsivos. Além disso, tem propriedades físicas, mecânicas e químicas semelhantes às de alguns tecidos do corpo humano. Tais propriedades permitem que ocorra a adesão e proliferação celular na presença deste material. A combinação de quitosano com agarose teve como objetivo explorar as interações poliméricas e o carácter termoresponsivo da agarose na produção de um hidrogel. Neste estudo a porosidade, capacidade de absorção de água, hidrofilicidade, biocompatibilidade e a atividade bactericida do hidrogel foram caracterizadas por microscopia eletrónica de varrimento, determinação do angulo de contacto, microscopia confocal, ensaio de citotoxicidade, determinação da concentração mínima inibitória e formação de biofilmes. Os resultados obtidos revelaram que o hidrogel promove a adesão e proliferação celular e possui atividade antimicrobiana. O desempenho do hidrogel na cicatrização de feridas foi avaliado através de ensaios *in vivo,* em que se registou que a cicatrização ocorreu num menor período de tempo e que os animais tratados com o hidrogel não evidenciavam qualquer reação inflamatória local ou sistémica.

Os diferentes estudos efetuados ao longo do meu doutoramento permitiram a produção/desenvolvimento de diferentes hidrogéis constituídos por polímeros naturais, que se pretendem aplicar na cicatrização de feridas. Os ensaios *in vitro* e *in vivo* revelaram resultados promissores que podem permitir a utilização dos hidrogéis desenvolvidos como substitutos de pele num futuro próximo.

Palavras-chave

Cicatrização; Ensaios *in vitro* e *in vivo;* Fatores de crescimento; Hidrogéis; Micropartículas; Polímeros naturais; Substitutos de pele

Abstract

The skin is the largest organ in mammals and acts as a barrier between the human body and the surrounding environment. It protects the underlying organs and defends the body against nocive agents.

After an injury, skin integrity is compromised and the organism triggers the wound healing process for restoring the structure and functions of this organ. Wound healing is an extremely dynamic and interactive biological process that involves extracellular matrix molecules, soluble mediators, various resident cells (fibroblasts and keratinocytes) and infiltrating leukocyte subtypes which, together, act to re-establish the integrity of the damaged tissue and replace the lost one.

To improve patients odds of survival and to minimize the loss of skin vital functions, this tissue has to be covered immediately after being damaged. Such highlights the importance of developing new wound dressings that improve the healing process, making it less painful and, simultaneously, contributing for the re-establishment of skin structure and functions in a shorter period of time.

In recent years, various dressings have been developed, using natural or synthetic materials, for restoring skin native properties and structure. Although, none of them is capable of fully accomplish this objective.

Taking this into account, the main objective of this work was to develop and characterize skin substitutes to be used in the treatment of skin disorders. Natural polymers such as agarose, chitosan and dextran were used for dressings production.

Initially, we evaluated the applicability of a chitosan hydrogel as a wound dressing. The morphology of the developed system was characterized by scanning electron microscopy while its cytotoxic profile and degradation by-products were evaluated through *in vitro* assays. *In vivo* experiments were also performed to evaluate the potential of the chitosan hydrogel for the treatment of skin burns. The results obtained revealed that the hydrogel developed has the required properties for biomedical application intended, once the *in vitro* and *in vivo* assays revealed that chitosan hydrogel and its degradation by-products are biocompatible and possess the ability to promote the healing of skin wounds.

In the second study, a dextran hydrogel was loaded with chitosan microparticles containing epidermal and vascular endothelial growth factors for the improvement of wound healing process. The hydrogel morphology and cytotoxicity profile and degradation by-products were characterized by scanning electron microscopy and *in vitro* assays. Furthermore, *in vivo* experiments were also performed to evaluate the applicability of the hydrogel for wound healing. The results obtained, revealed that the animals treated with this hydrogel showed a faster wound healing with no signs of local or systemic inflammatory response. Moreover, a unique application per week of this skin substitute allowed a faster healing than that obtained when growth factors were topically applied in the wound every two days. Dextran hydrogel

proved that it can be used as a wound dressing and also as a carrier of microparticles containing growth factors involved in wound healing.

In the third study, a thermoresponsive chitosan/agarose hydrogel was produced for being used in the wound healing process. The porosity, wettability, hydrophilicity, biocompatibility, and bactericidal activity of the hydrogel were characterized by scanning electron microscopy, studies of water uptake, determination of contact angle, confocal microscopy, cytotoxic assays, determination of minimum inhibitory concentration and biofilm deposition, respectively. The performance of this polymeric matrix in the wound healing process was evaluated through *in vitro* and *in vivo* assays. The attained results revealed that the hydrogel promotes cellular adhesion and proliferation and also its bactericidal activity. The *in vivo* studies showed also an improved healing and the lack of a reactive or a granulomatous inflammatory reaction in the skin lesions treated with this hydrogel.

During this PhD, various natural polymers were used to produce three different hydrogels, aimed for wound healing. The *in vitro* and *in vivo* assays revealed promising results that may allow their use as skin substitutes in a near future.

Keywords

Growth Factors; Hydrogels; *In vitro* and *in vivo* studies; Microparticles; Natural polymers; Skin substitutes; Wound healing.

Thesis Overview

This thesis is organized in six main chapters.

The first chapter includes the overall objectives established for the development of the work plan defined for the PhD. The second chapter presents a literature review, where the state of the art of skin disorders and the different skin substitutes, currently available, are presented. Thereafter, the third, fourth and fifth chapters present the results obtained during the development of this PhD research work plan, that were summarized in some original research papers published in international peered review journals, and are organized as follows:

Chapter 3 – Development of a new chitosan hydrogel for wound dressing;

Chapter 4 - Dextran based-hydrogel containing chitosan microparticles loaded with growth factors to be used in wound healing;

Chapter 5 - Thermoresponsive chitosan-agarose hydrogel for skin regeneration;

Finally, in the sixth chapter has the concluding remarks summarize all the main achievements attained during this PhD and also presents some future perspectives of the work that could be done in this area of research.

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Chapter 1
Global aims

Global aims

The principal objectives of this doctoral work plan were the development and characterization of new skin substitutes to be used in the treatment of skin disorders, since, to date, none of the currently available skin substitutes fully restores all the native properties of the skin. According to these global aims, the work was developed respecting the following intermediate tasks:

1. Production of skin substitutes to promote the wound healing, using natural polymers such as agarose, chitosan and dextran, with/without the incorporation of growth factors in their 3D polymeric structure.

2. Characterization of the morphology and porosity of skin substitutes.

3. Evaluation of the biocompatibility of skin substitutes (both by *in vitro* and *in vivo* studies) in order to study the suitability of the material for the aimed biomedical application.

Chapter 2
Introduction

2. Introduction

2.1. Skin

Skin is the outermost barrier of human beings and the largest organ of the body, covering about 1.7 m^2 and accounts for approximately 16% of body total weight [1-5].

Several authors consider that the human skin is organized in three anatomical distinct layers (figure 2.1) that under normal physiological circumstances are self-renewable [2, 6-8]. From outermost to innermost the three layers found are: (i) epidermis: aneuronal and avascular multilayered that is constantly being renewed; (ii) dermis: a supporting layer that has a remarkable capacity for retaining water; (iii) hypodermis: a sub-layer rich in fat that provides insulation, mechanical cushioning and energy store [2, 3]. Moreover, skin has numerously appendages such as hair follicles, and sebaceous, eccrine and apocrine glands. These appendages are combined with blood vessels, nerves, and pressure and touch receptors [2-4].

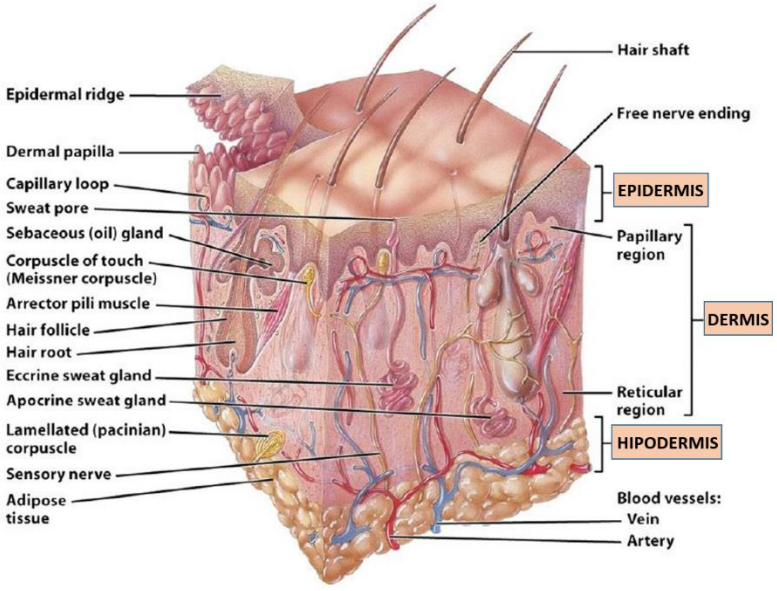


Figure 2.1: Illustration of the structure of the human skin, where epidermis, dermis and hypodermis are identified (adapted from [7]).

2.1.1. Skin structure

2.1.1.1. Epidermis

In utero the fetus has no need for a skin barrier, so the epidermal layer does not start to develop until the 24th week of pregnancy. A completely developed skin is only attained after 34 weeks of fetus gestation [9]. However, skin barrier function is not established until the early stages

of postnatal life. The immature epidermal barrier in infants is responsible for a higher disease susceptibility and also an increased permeability to exogenous substances [10].

The epidermis is a thin and highly cellular layer, composed mainly by keratinocytes (90–95%), melanocytes, Merkel cells and also by specialized dendritic Langerhans cells. Despite, its thickness, this layer is also capable of providing a physical, chemical/biochemical and an adaptive immunological barrier to the human body [9, 11, 12].

The formation of epidermis involves a continuous process of cell proliferation, differentiation and, ultimately, death and shedding that allows its compartmentalization into a number of layers, that represent the different stages of keratinocyte maturation. Due to this fact, the epidermis may be subdivided, from the inside to the outside, into five layers: the *Stratum Basale, Spinosum, Granulosum, Lucidum* and *Corneum* (figure 2.2) [9].

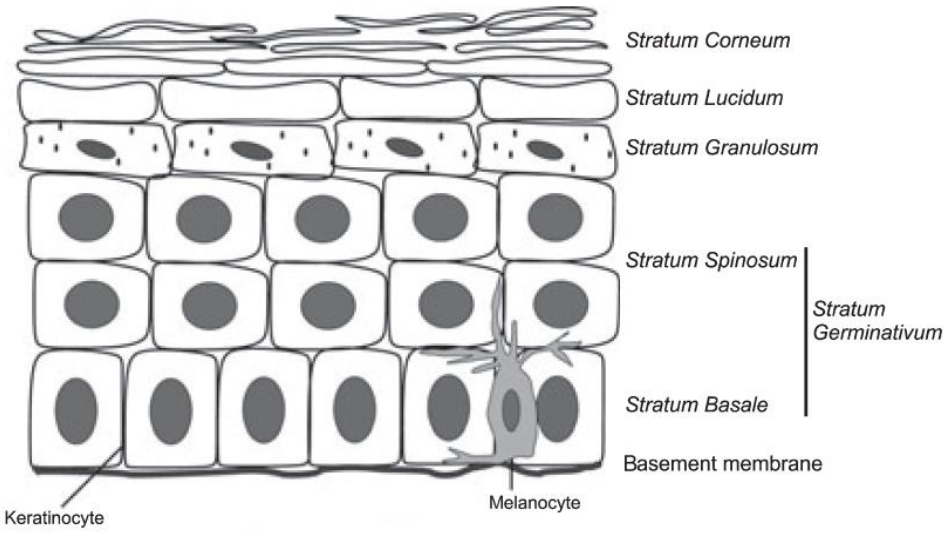


Figure 2.2: Illustration of epidermis layers (adapted from [9]).

- *Stratum basal*

The basal cell layer is comprised mostly of keratinocytes that are in constant division. They are responsible for the continuous self-renew of epidermis. When a new layer of cells is formed they replace the older ones, by moving outward and suffering changes in shape and chemical composition. These cells become keratinized and consequently die. Furthermore, the *stratum basal* is also composed by melanocytes (involved in pigment-production) and Merkel cells (which are thought to act as skin sensors).

- *Stratum Spinosum*

This layer contains Keratinocytes that are constantly growing and are involved in the early keratin synthesis. Langerhans cells (that are involved in the skin immune defence system) are mostly found in this layer.

- *Stratum Granulosum*

Is formed by a granular cell layer composed of differentiated keratinocytes that become flattened, lose their nuclei and produce keratohyalin granules.

- *Stratum Lucidum*

This *stratum* is a thin and clear/translucent layer of dead cells that is only seen in palms and soles.

- *Stratum Corneum*

The end result of keratinocyte maturation can be found in this layer. Keratinocytes are completely transformed in corneocytes, which are dead cells that do not present nuclei and organelles.

The cellular progression from the basal layer to the skin surface takes about 30 days [6].
Epidermis and dermis are separated by the extracellular matrix (ECM), also known as the basal lamina [9], that can be divided into *lamina lucida* (the layer that is closer to the epidermis and that is constituted by laminin, integrins, entactins and dystroglycans) and *lamina densa* (a sheet-like structure that is mainly composed of collagen type IV) [6].

2.1.1.2. Dermis

The dermis is the layer between epidermis and hypodermis and consists primarily on an ECM composed of: (a) collagen fibres, that provide tensile strength to skin; (b) elastin, which confers elasticity and resilience to normal human skin; (c) non-collagenous glycoproteins, such as fibrillins, fibulins and integrins, which are involved in matrix three-dimensional (3D) organization and also promote cell-matrix interactions; and (d) proteoglycan/glycosaminoglycan macromolecules, that are involved in the hydration of skin [13]. Furthermore, dermis is vascularized and its blood capillaries supply the epidermis with nutrients and perform gases exchanges [6]. Dermis layer is composed by three main types of cells: fibroblasts, macrophages and adipocytes [6].
Dermis is usually subdivided into two layers: the superficial layer immediately under the epidermis is called papillary dermis, while the deeper and thicker connective tissue that is called reticular dermis [6, 14, 15].

- Papillary dermis

It is a thin layer with rete pegs and ridges along the dermal-epidermal junction that increase the surface contact area between the epidermis and the dermis. This layer contains a terminal network of blood capillaries and mechanoreceptors of encapsulated Meissner corpuscles,

rapidly adaptive receptors, being sensitive to tactile stimuli of slight deformations in the epidermis.

- Reticular dermis

It is a deeper and thicker connective tissue composed by a high concentration of collagenous, elastic and reticular fibers, supporting the strength, extensibility, and elasticity of this layer. Within this region, roots of skin appendages are distributed.

2.1.1.3. Hypodermis

Hypodermis, as the deeper layer of skin, is well vascularized and is composed mainly by adipose tissue, about half of the total fat stored in the body is localized in this layer. However, their amount varies with age, sex and food intake. This layer, besides being involved in the connection between skin, bones and adjacent muscles, is also determinant for the thermoregulatory and mechanical properties of the skin [11].

2.1.1.4. Skin appendages

Skin appendages, such as sweat and sebaceous glands, hair follicles and nails are associated with all the layers of the skin. They have different functions based on their structures. Sweat glands regulate body temperature by the secretion of sweat onto the surface of the skin. Sebaceous glands secrete sebum to moisturize the skin and hair. Nails confer protection to the distal phalanx and the fingertip [6]. Hair follicles play an important role in wound healing, once the epidermal basal layer constitutes the outer cell layer of these structures and it has been shown that such basal cells, present in hair follicles, can move out and repopulate the epidermis after healing [16].

2.1.2. Functions played by skin

The organization of the skin into epidermal, dermal and hypodermal layers is responsible for the thickness, strength, and flexibility that this organ presents and by the different functions that it is able to perform [9]. Skin most important function is to provide an effective barrier between the ‘inside’ and the ‘outside’ of the organism, acting as a shield to protect the organism against external insults (eg. pathogens, microorganisms and toxic chemicals), prevent dehydration and also maintain the body temperature [2, 12, 17]. Besides the skin role in physical, immunological and metabolic protection, this organ is also involved in the synthesis of vitamin D when exposed to sunlight. Vitamin D has an important role in the maintenance of

calcium homeostasis and in the protection of skin from hazardous effects of ultraviolet (UV)-radiation [18, 19].

Furthermore, human skin, besides conferring protection, also provides a potential entrance for functional active drugs/reagents through topical delivery and/or transdermal delivery (like for the case of vaccines administration). Three routes are known for the transport of bioactive molecules across the skin: a) intercellular pathway, if the biomolecules diffuse through the intercellular lipid domains; b) intracellular pathway, if the biomolecules penetrate through the keratinocytes, and then across the intercellular lipids; c) transappendageal pathways, if the biomolecules use the skin appendages (eg. follicular) to penetrate the skin, and do not need to cross the *stratum corneum* (figure 3)[6, 20].

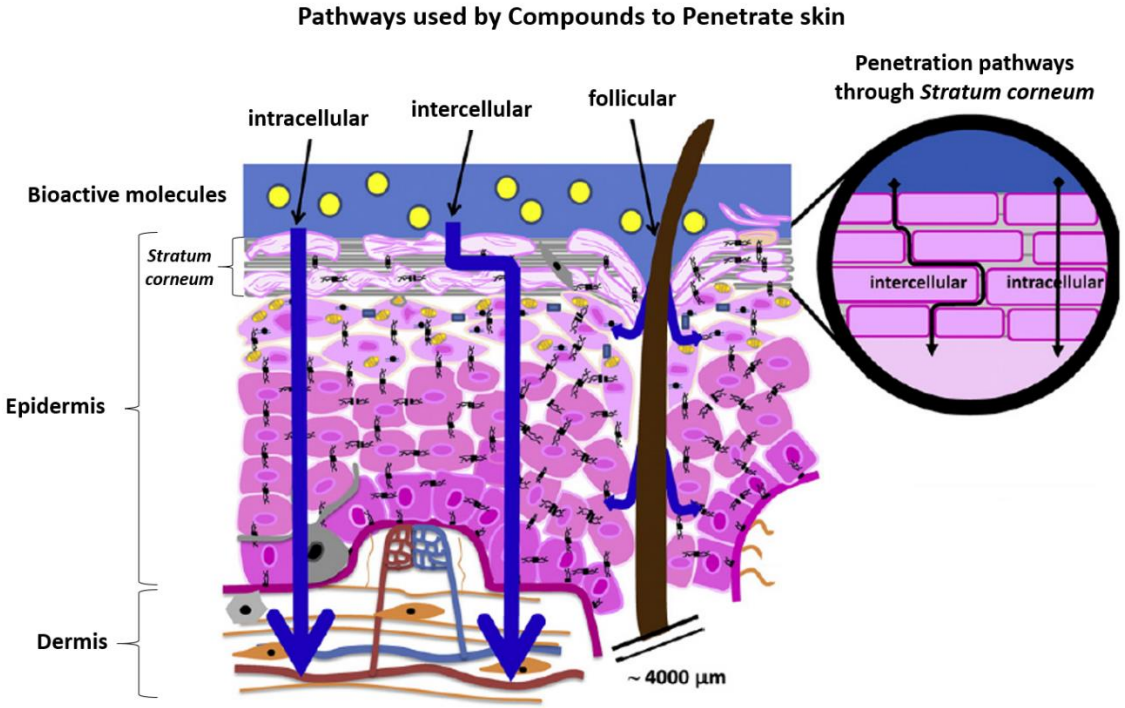


Figure 2.3: Scheme of the pathways used by drugs/bioactives molecules to penetrate through into the skin (adapted from [20]).

2.2. Wounds

Once the external barrier is breached and skin integrity is compromised, innate surveillance mechanisms set off a cell-signalling cascade that limits pain, controls infection, and improves the wound healing mechanism, ultimately creating a scar [2, 6, 21]. Skin importance is highlighted when large areas of this tissue are lost, as a consequence of diseases or injuries (as in the case of extensive burns), resulting in significant morbidity and mortality [22].

The Wound Healing Society defines wound as being the result of disruption of normal anatomic structure of this organ and also its functions [23].

Any disruption of skin integrity can lead to an injury or illness that may result in a substantial physiological imbalance, and ultimately in significant disability or even death [24]. Thus, the disruption of the skin by wounding can lead to fluid loss, infection, scarring, hypothermia, compromised immunity and change in body image. Such occurrences demand a quick re-establishment of skin structure and functions [25, 26].
Wounds can be classified into 2 broad types: chronic and acute. Chronic wounds, do not heal orderly and need more time to heal. By turn, acute wounds, can be repaired through an orderly and timely process [26-28].

2.2.1. Chronic Wounds

Chronic wounds heal by a process that is incomplete and disturbed by several factors, including absence of clot formation (which reduce the levels of active/vital growth factors in the wound environment), and bacterial colonization that triggers a high immune response for removing the debris (debridement can improve healing by exposing the viable tissue), leading to an increase in the healing time [26, 29].
Chronic wounds frequently occur in individuals who have underlying comorbidities, including advanced age, peripheral blood vascular disease, obesity, diabetes, chronic steroid use, or other chronic diseases that impair tissue healing [26, 30]. All these facts have a relevant influence on the healing process, being a major concern considering the ageing demographic pressure and the increasing of the obesity of the worldwide population. Pressure, venous insufficiency, diabetic foot ulcers and ischemic wounds are the most prevalent types of chronic wounds [30].

2.2.2. Acute Wounds

Acute wounds are characterized by a disruption of skin layers through a simple shearing force or an object. Acute wounds heal through a normal, orderly, and timely reparative process that results in a sustained restoration of the anatomic and functional integrity of this organ [26]. Acute wounds can be categorized based on causes such as: burns, abrasion, punctures, incisions and gunshots [26, 31].

2.2.2.1. Burns

The World Health Organization estimates that, annually, over 300 000 deaths are caused to fire-related burn injuries. Burn cause damages in the skin and underlying structures due to excessive heat or chemicals agents. Furthermore, they have also devastating physical and emotional consequences to the patients [2].

Burn severity is determined by patient age and health condition, depth, position and size of the burned area. Babies and the elderly have a higher mortality rate than older children, young and middle-aged adults. Moreover, sick patients have also a higher mortality rate when compared to healthy patients. The depth of heat injury depends upon the quantity of heat exposure and depth of heat penetration. The localization of the burn in the body also affects its severity, i.e. regions where thinner skin is present the burn will be deeper [32].
Burns are usually divided into five degrees of depths (figure 2.4), based on the number of layers and the area of skin affected [33-35]:

- Superficial or epidermal burns, typified by the common sunburn and are usually confined to the epidermis;
- Superficial partial thickness burns, involve the destruction of the epidermis and the upper part of the dermis (Blisters are included in this type of burns);
- Deep partial thickness burns, where epidermis and most of the dermal layer is destroyed. As a result, the wound is white and dry;
- Full-thickness burns involve epidermis, dermis, and epidermal appendages destruction. This burn is waxy and white;
- Subdermal burns involve the destruction of the outer epidermis, dermis and it is extend to the tissue below, including fat tissue, tendons, muscles, and bones.

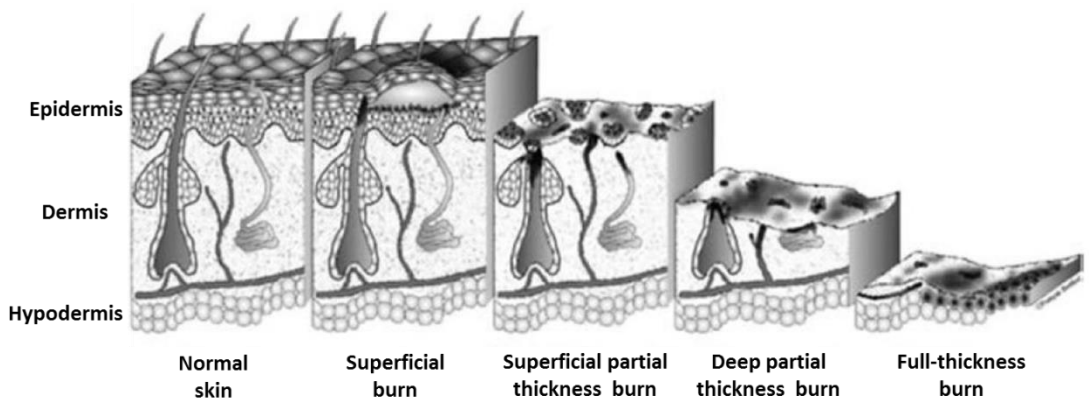


Figure 2.4: Representation of the different degrees of burn severity (adapted from [35]).

After a trauma or injury the wound healing process starts through an organized and complex cascade of biological pathways, in order to restore the tissue integrity and homeostasis [36, 37].

2.3. Wound Healing

The wound healing is an innate process that starts immediately after the body suffers an injury [25]. The main goals of this process are to achieve a rapid wound closure and a functional and aesthetic scar [38]. After trauma, several intracellular and intercellular pathways must be

activated and coordinated to restore skin structure and functions [39]. These pathways are specific, dynamic and interactive involving soluble mediators (eg. cytokines and growth factors), blood cells (eg. platelets and neutrophils), extracellular matrix and parenchymal cells (keratinocytes and fibroblasts) [40].

The wound healing process progresses through a series of five interdependent and overlapping stages designated by haemostasis, inflammation, migration, proliferation and remodelling phases (see figure 2.5) [23]. Each stage must happen in a precise and controlled manner, which is crucial for the wound healing process to occur. Interruptions, aberrancies, or prolonged healing process can lead to a delayed wound healing or a non-healing [41].

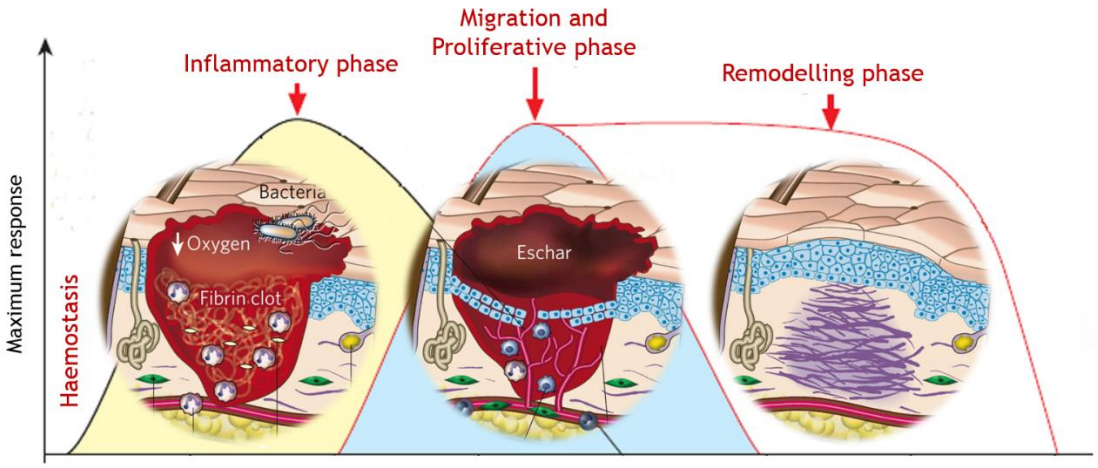


Figure 2.5: Illustration of the phases of the wound healing process (adapted from [39, 42]).

2.3.1. Wound healing phases

2.3.1.1. Haemostasis and Inflammation

After a tissue injury, the disruption of blood vessels occurs and consequently an extravasation of blood constituents [25, 39, 42]. Bleeding typically occurs when the skin is injured and it allows the removal of bacteria and/or antigens from the wound. In addition, bleeding activates the first step of wound healing, the haemostasis [23], where platelets are the first to arrive after injury occurrence and play a vital role in normal haemostasis. They suffer adhesion as well as aggregation to the wall of the injured blood vessels and to the exposed collagen within the ECM. Simultaneously, they release cytokines, growth factors (GFs) and numerous pro-inflammatory mediators (see table 2.1 and 2.2) that promote fibrin clot formation and vascular constriction [25, 31, 42]. The formation of a clot serves as a temporary shield that protect the exposed wound tissues [43].

Table 2.1: Principal growth factors involved in wound healing.

Name	Major Source	Functions related to wound healing	Ref.
Vascular Endothelial Growth Factor (VEGF)	• Endothelial cells • Fibroblasts • Macrophages • Neutrophils • Platelets • Smooth muscle cells	• Chemotactic agent • Increases vascular permeability • Promotes endothelial cell migration and proliferation • Promotes formation of granulation tissue • Stimulates the angiogenesis	[44]
Fibroblast Growth Factor (FGF)	• Endothelial cells, • Fibroblasts, • Keratinocytes • Macrophages • Smooth muscle cells	• Induces angiogenesis • Regulates the production of collagen • Stimulates proliferation and/or migration of the major cell types involved in wound healing, including endothelial cells, fibroblasts and keratinocytes	[45]
Epidermal Growth Factor (EGF)	• Fibroblasts • Macrophages • Platelets	• Promotes the formation of granulation tissue • Stimulate differentiation, proliferation, migration and adhesion of keratinocytes • Stimulates of fibroblast motility	[46]
Keratinocyte Growth Factor (KGF)	• Fibroblasts	• Stimulates proliferation and migration of keratinocytes	[36]
Platelet-Derived Growth Factor. (PDGF)	• Endothelial cells • Fibroblasts • Macrophages, • Platelets	• Induces the proliferation of fibroblasts and thus the production of ECM • Promotes the re-epithelisation • Stimulates mitogenicity and chemotaxis of neutrophils, macrophages and fibroblasts	[47]
Transforming Growth Factor-β (TGF-β)	• Fibroblasts, • Keratinocytes, • Macrophages, • Platelets	• Induces the re-epithelialization • Promotes granulation tissue formation • Stimulates matrix formation and remodelling	[48]
Transforming Growth Factor-α (TGF-α)	• Fibroblasts • Keratinocytes • Lymphocytes • Macrophages • Platelets	• Promotes the formation of granulation tissue • Stimulates the proliferation of epithelial cells and fibroblasts	[49]

Table 2.2: Principal pro-inflammatory mediators involved in wound healing.

Name	Major Source	Functions related to wound healing	Ref.
Tumour Necrosis Factor (TNF-α)	• Macrophages • Neutrophils	• Inflammation • Reepithelialization	[50]
Interleukin-1 (IL-1)	• Keratinocytes • Macrophages • Monocytes • Neutrophils	• Activates fibroblasts • Increases keratinocyte migration and proliferation	[51]
Interleukin-6 (IL-6)	• Macrophages • Neutrophils	• Chemoattractive to neutrophils • Stimulates the proliferation of keratinocytes	[51]
Insulin-like Growth Factor (IGF-1)	• Fibroblasts	• Promotes fibroblast proliferation • Stimulates synthesis of sulphated proteoglycans and collagen	[52]
Hepatocyte Growth Factor (HGF)	• Endothelial cells • Fibroblasts, • Keratinocytes	• Induces the re-epithelialization • Promotes Neovascularization • Stimulates the formation of granulation tissue	[53]

The inflammatory phase arises almost simultaneously with haemostasis (figure 2.6), starting from within a few minutes of injury to 24 h and lasts for about 3 days. It involves both cellular and vascular responses [23].

In the inflammation phase the neutrophils are initially attracted to the clot area by various chemotactic agents such as N-formyl peptides released by bacteria [54]. They begin to adhere to the endothelial cells in the adjacent blood vessels (margination) and start to move through the vessel wall (diapedesis) and accumulate within the wound area [25, 42]. The presence of the neutrophils is crucial within the first days after injury, due to their ability to perform phagocytosis and secret proteases that kill bacteria and help in necrotic tissue degradation process [55]. They act as 'wound cleaners' and provide the first line of defence against infection [26, 41, 56]. After 2/3 days, neutrophils begin to disappear from the wound bed suffering apoptosis. During this period, basal cells, at the cut edge of the epidermis, begin to exhibit increased mitotic activity [25, 42].

Subsequently, monocytes that have been progressively infiltrated into the wound bed are activated to become macrophages, usually around 48 to 96 hours after injury, that act as phagocytic cells (second line of defense). Macrophages have high phagocytic activity and remove all non-viable cells, bacteria-filled neutrophils, damaged extracellular matrix, debris and any bacteria from the wound site. Furthermore, they are also involved in the production of numerous enzymes (collagenases involved in debride the wound), cytokines (IL-1 and IL-6 that stimulate the re-epithelialization) and growth factors such as: EGF (that stimulates the re-epithelialization) TGF- ß, FGF and PDGF (which promote cell proliferation and the synthesis of ECM molecules by resident skin cells) and VEGF (that stimulates the angiogenesis and granulation). They are also responsible for attracting fibroblasts and smooth muscle cells into the wound site [25, 31, 55-57]. Thus, macrophages, have a pivotal role in the transition between inflammation and next phase of wound healing, the migratory phase [40, 55].

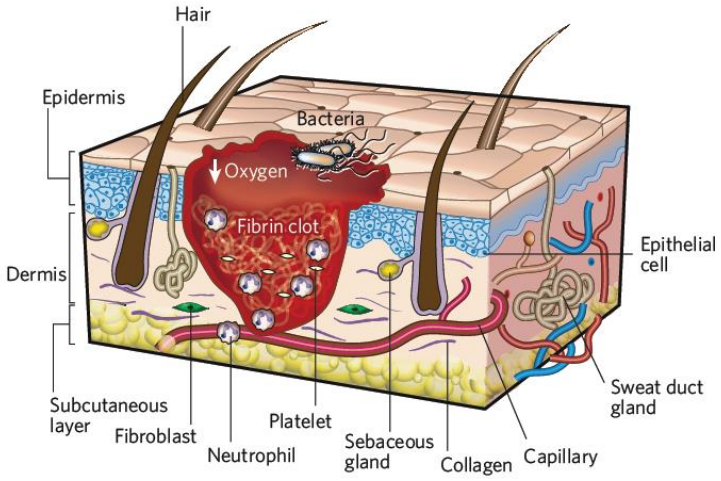


Figure 2.6: Representation of inflammatory phase of the wound healing process (adapted from [39]).

2.3.1.2. Migration and Proliferative Phase

The migration phase is the final stage of visible wound healing process. This phase involves the migration of keratinocytes, fibroblasts and endothelial cells to the injured area for replacing the damaged and lost tissue (see figure 2.7) [23, 25].

The proliferative phase (approx. 2–10 days after wounding) occurs almost simultaneously or just after the migration phase and basal cell proliferation [23]. The main objectives of this phase are the covering of the wound surface, formation of granulation tissue and the reconstruction of the vascular network [41, 42, 55].

Fibroblast, keratinocyte and endothelial cells are the principal types of cells involved in the proliferative phase, since they support the re-epithelialization, capillary growth, collagen production, and formation of granulation tissue at the injury site [41].

Re-epithelialization is a process that allows to re-establishment of the epidermis structure after cutaneous injury [31]. This process is ensured by keratinocytes at the wound edges and by epithelial stem cells from hair follicles or sweat glands [55]. Keratinocytes are responsible for edge proliferation and maturation, depositing components of basement membrane (mainly collagen type IV and VII and laminin), and finally by restoring the stratified epithelium [31, 39, 42].

Another important step of this phase is the restoration of the vascular system of the skin, a process known as angiogenesis [25, 31, 39, 41, 55]. Angiogenesis is a complex process that depends on ECM of the wound bed, as well as of the migration and mitogenic stimulation of endothelial cells [40]. These events allow the wound bed reconnection to the nutritive perfusion system [55].

Then, endothelial cells proliferate and migrate into the wound to build sprouts that form the temporary vessel. Thereafter, the new vessels differentiate into arteries and venules and subsequently, stabilize their vessel wall by recruiting pericytes and smooth muscle cells. Then, the blood flow is re-established completing the angiogenic process [55]. The most important positive regulators of angiogenesis are VEGF and FGF [39, 40, 58].

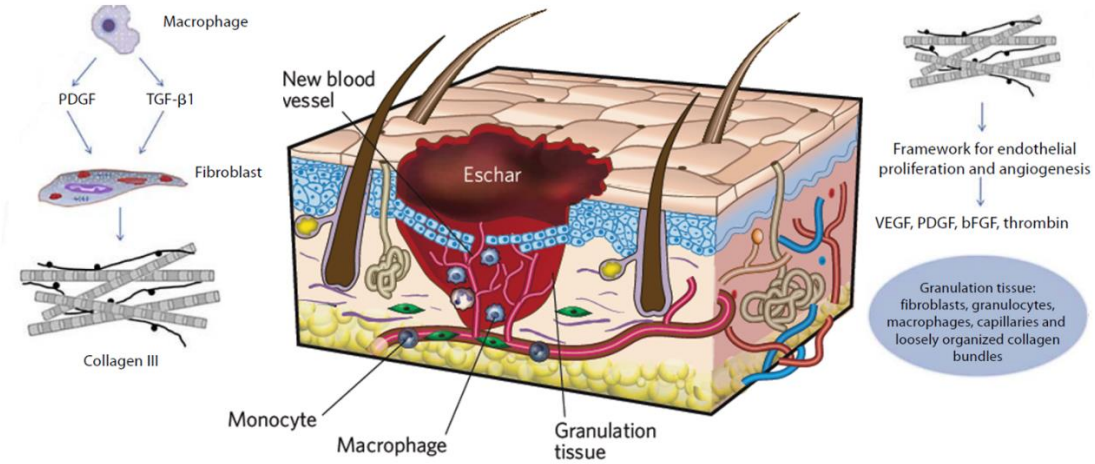


Figure 2.7: Illustration of migratory and proliferative phases of wound healing process.(adapted from [39, 55]).

The next stage of this phase is the development of the acute granulation tissue [55]. The dominating cells in this phase are the fibroblasts, that are involved in the synthesis of collagen type III and ECM constituents (i.e. fibronectin, glycosaminoglycans, proteoglycans and

hyaluronic acid) [25, 31, 42, 55, 56]. The formation of the ECM is crucial for providing support for cell migration and adhesion. Furthermore, fibroblasts organize and regulate the cell growth, migration and differentiation within the wound [31, 55]. At the end of this phase, fibroblasts are stimulated by macrophages, and some of them become differentiated in to myofibroblasts [39]. Myofibroblasts are contractile cells that re-join the borders of the wound [31, 36, 39-41, 55].

2.3.1.3. Remodelling Phase

Remodelling is the last phase of wound healing and it begins 2-3 weeks after injury and lasts for a year or more [39, 55, 59]. During this phase, the formation of granulation tissue stops through the apoptosis of the endothelial cells, macrophages and myofibroblasts, or with their exodus from the wound [39, 55]. Moreover, the ECM components will be replaced by the balanced mechanisms of proteolysis and new matrix secretion, accompanied by a gradual decrease of wound vascularization [59]. One of the most important changes in wound remodelling process is the gradual replacement of immature collagen type III by mature collagen type I (figure 2.8) [31, 39, 42, 55, 59]. This modification is tightly controlled and mainly performed by matrix metalloproteinases (MMPs) that are secreted by fibroblasts, macrophages and endothelial cells. This remodelling process increases the strength of the repaired tissue [31, 39, 42].

In the final stage, the granulation tissue evolves into an avascular scar that is composed of inactive fibroblasts, dense collagen, fragments of elastic tissue and other components of the ECM [42]. Over a period of 1 year or more, the scar matures and the wound site reacquires a stable pre-injury phenotype. During this stage the fibronectin and hyaluronan are broken down and collagen bundles acquire a higher diameter, increasing tensile strength of the skin. Tensile strength, is a functional assessment of collagen that increases up to 40% of the initial strength of skin at 1 month, and may continue to increase for 1 year, reaching up to 70-80% of its pre-injury strength [31, 42].

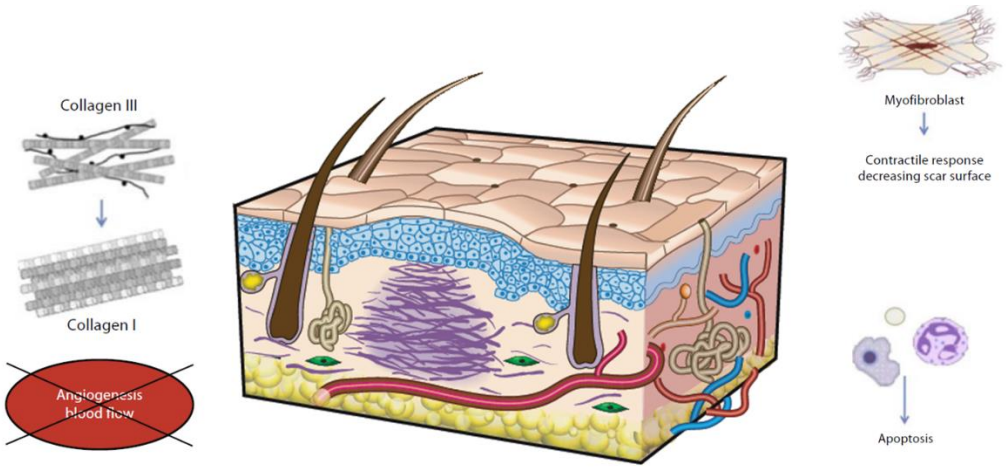


Figure 2.8: Illustration of remodelling phase of the wound healing process (adapted from [39, 55]).

2.3.2. Wound Exudate

Wound exudate, also known as wound fluid or wound drainage, is derived from plasma that leaks from damaged tissue [60]. It is a derived of the blood where most of the red cells and platelets have been removed [23]. Exudate plays an important role in all the stages of wound healing, irrigating the wound continuously, keeping it moist, enabling diffusion of immune and GFs, assisting autolysis and transporting essential nutrients for cell metabolism. Despite the necessary and desirable, synthesis of exudate to support wound healing, when exudate becomes excessive, problems arise [23, 60]. The excess exudate is a consequence of the edema caused by inflammation, reduced mobility, venous or lymphatic insufficiency and autolytic debridement [23].

2.3.3. Abnormal wound healing

Abnormal wound healing encompasses a wide spectrum, from chronic wounds to hypertrophic and keloidal scars [42, 59]. The healing of chronic wounds is compromised by local and systemic factors. Local factors involve those that directly affect the characteristics of the wound itself such as, infection and oxygenation (table 2.3) [41, 42].

Table 2.3: Local factors that influence wound healing [41, 42].

Factors	Influence in the wound healing
Infection	Bacteria and endotoxins can lead to a prolonged increase of pro-inflammatory cytokines and extend the inflammatory phase. In these situations, the wound may enter a chronic state and fail to heal
Oxygenation	In wounds where oxygenation is not restored, healing is delayed and impaired

Systemic factors involve characteristics such as age, alcoholism, diabetes, medications, nutrition, obesity, sex hormones and smoking (table 2.4) [23, 41, 42].
In cases of excessive wound healing, hypertrophic and keloidal scars are formed as a result of the overproduction of all components of the healing process, such as fibroblasts, collagen, elastin and proteoglycans [42]. In these conditions, abnormalities occur in inflammation, cell migration, proliferation and remodelling of the wound matrix [40]. Another type of scars, classified as atrophic, cause a valley or depression in the skin, due to collagen destruction during the course of the inflammatory process of skin diseases like cystic acne or varicella [8, 61].

Table 2.4: Systemic factors that influence wound healing [41, 42].

Factors	Influence on the wound healing process
Age	The age-related changes in healing capacity demonstrate that every phase of healing undergoes characteristic age-related changes, including: - decreased secretion of growth factors, - decreased wound strength, - delayed angiogenesis and collagen deposition, - delayed infiltration of macrophages and lymphocytes, - delayed re-epithelialization, - enhanced platelet aggregation, - impaired macrophage function, - increased secretion of inflammatory mediators, - reduced collagen turnover and remodelling.
Alcoholism	Acute alcohol exposure causes: - changes in the protease balance at the wound site, - impairing of the early inflammatory response, - inhibition of wound closure, angiogenesis, and collagen production.
Diabetes	The impaired healing that occurs in individuals with diabetes involves: - decreased host immune resistance, - dysfunction of fibroblasts and epidermal cells, - high levels of MMPs, - hypoxia, - impaired angiogenesis and neovascularization, - neuropathy.
Medications	Many medications (eg. glucocorticoid steroid and non-steroidal anti-inflammatory drugs), such as those that interfere with clot formation or platelet function, inflammatory responses and cell proliferation, affect wound healing.
Nutrition	Proteins, carbohydrates, polyunsaturated fatty acids, vitamins A, C and E, magnesium, copper, zinc, and iron play a significant role in wound healing, and their deficiencies delay this process.
Obesity	In obese patients some local conditions impair the wound healing, promoting wound complications such as: - decreased vascularity in adipose tissue, - friction caused by skin on skin, - increased wound tension, - increased tissue pressure, - hematoma and seroma formation, - venous hypertension.
Sex hormones	Estrogens can improve the age-related impairment in healing of both men and women, while androgens regulate cutaneous wound healing negatively.
Smoking	Tobacco smoke promotes several modifications on wound healing process, such as: - affects several cell types (eg. monocytes, macrophages and fibroblasts) and processes that are important to healing, - decreases fraction of oxygenated hemoglobin in the bloodstream, - causes tissue ischemia, since there is a decreased blood flow to the affected tissue.
Stress	Psychological stress causes a substantial delay in wound healing.

2.4. Clinical Management of Wounds

In order to minimize/solve the problems associated to non-healing wounds, it is essential re-establish the outmost barrier after an injury, i.e., cover it in order to avoid bacterial contamination, reduce water/blood loss and control pain [62]. In order to promote the wound healing, different treatments were developed and are being applied in clinical practice. Treatments such as, debridement, negative pressure wound therapy, hyperbaric conditions, topic application of growth factors, skin grafts and wound dressings are the cutting edge among the treatments currently applied [63].

2.4.1. Skin grafts

Skin grafts, have been used, for over a century, to cover the surface of this tissue when an injury occurred [64]. The skin grafts can have different origins. Traditionally the most used are the autografts, allografts and xenografts. Autografts are obtained from the patient himself and present a higher healing success rate. Although, they have a limited supply and obtaining it has associated a donor site morbidity. Allograft skin can be gotten from a family member or another living volunteer from the same species, but it is commonly harvested from cadavers. These grafts are abundant, but have always associated the risk of disease transmission and immunological rejection [64-66]. Besides autografts or allografts, xenografts emerged as possible alternatives of skin substitutes that are gathered from animals of different species. However, xenografts have some drawbacks associated like the possibility of viral, prion-mediated and bacterial infections and also immunological rejection similarly to that determined for allografts [65].

2.4.2. Traditional skin substitutes

In the past, traditional or inert/passive dressings such as natural or synthetic bandages, cotton wool, lint and gauzes (all with varying degrees of absorbency) were used for the management of wounds. They maintained the wound dry, by allowing the evaporation of wound exudates, and prevent the entry of harmful bacteria into the wound [23]. These traditional dressings affect the wound healing, since they do not have the properties of an ideal wound dressing that are fundamental for the wound healing process. In the last two decades, new types of dressings have been emerged in order to overcome some of the risks/problems associated with these traditional dressings [23, 67].

Despite the enormous number of dressings, there is not a single one that is able to fully re-establish skin native properties and/or be suitable for the treatment of all types of wounds. Moreover, different dressings are required along the healing process [23, 63, 68]. An ideal

dressing must possess different properties such as: be absorbent; afford pain relief; comfortable to wear; debriding; having physical and mechanical characteristics that allow them to restore the functions of normal skin; haemostatic; hypoallergenic; inexpensive; maintain a moist environment; non adherent to the wound surface; not shed fibers in the wound; nontoxic; protect the wound against environment and provide thermal insulation [68, 69]. The development of a wound dressing that corresponds to these requirements is a huge challenge for skin tissue engineering.

2.4.3. Biomaterials and wound dressings

An appropriate biomaterial is a key structural element to allow tissue regeneration since a provisional ECM is essential to support the complex repair processes required for skin healing [70, 71]. Biomaterials have been described, since the antiquity, as materials used for the production of medical devices [72]. Recently, Williams defined biomaterial as "a substance that has been engineered to take a form which, alone or as part of a complex system, is used to direct, by control of interactions with components of living systems, the course of any therapeutic or diagnostic procedure" [73].

Biomaterials are intended to treat, deliver, augment or replace the tissue or function of body that was affected either by disease or trauma [74].

Generally, an ideal biomaterial to be used in tissue engineering applications must have properties such as [70, 75-77]:

a. Biocompatibility, highlighting the importance of triggering an appropriate host response;
b. Biodegradability and bioresorbability if the material was provisional. Moreover, the degradation products must be removed from the body through metabolic pathways or its accumulation in the body cannot induce any inflammatory response or toxicity;
c. Promote cell adhesion, proliferation, migration and differentiation in order to promote the development of a functional tissue;
d. Their mechanical and physical properties must be compatible with tissues regeneration. These biomaterials can provide temporary mechanical support for cell proliferation and also lead with *in vivo* forces that are exerted by the surrounding tissue. The biomaterial role ends when the tissue is able to self-support;
e. The biomaterials surface-area-to-volume ratio should be high enough in order to allow the delivery of drugs and also sustain a high cellular density;
f. A highly porous 3D structure that allows tissue ingrowth and the transport of nutrients and gases exchange;
g. Be sterilized without losing their intrinsic properties.

Biomaterials are generally classified into three main categories: metals, ceramics, and polymers. Polymers offer a versatility that is unmatched by metals and ceramics, and have been extensively used in the production of wound dressings [78]. These polymers are frequently classified based on source (natural and synthetic). Natural materials are obtained from nature, while synthetic materials are produced in the laboratory [75, 79-81]. One of the most frequently used natural polymer, in wound dressing production, is collagen [82, 83], which can be obtain from a variety of organisms such as bovine, porcine and jellyfish [84]. Poly caprolactone (PCL) is an example of synthetic polymer used for wound dressings. PCL has been used as a carrier for bioactive molecules, such as antibacterial agents to infected wounds [85]. Another example of synthetic biomaterial is the Polyurethane that has been extensively studied as materials for wound dressing due to its semipermeability (impermeable to bacteria and water but permeable to gases) creating a moist environment desirable for wound healing [62].

The application of both natural and synthetic polymers presents clear benefits for skin regeneration [79, 86]. Natural polymers are able to match the 3D architecture (similarity to the ECM that support the growth, proliferation and differentiation of cells), allow biological recognition (presentation of receptor-binding ligands) and present susceptibility to cell-triggered proteolytic degradation and remodelling [71, 75, 77, 79, 87, 88]. However, natural polymers have some limitations, such as the degree of purity and batch-to-batch inconsistency, poor mechanical strength and variations in degradation rates [79, 89]. Another important disadvantage to consider is the immunogenicity and possibility of pathogen transmission, depending on their source [77, 89].

Although, some of these limitations can be overcame by chemical synthesis of peptides and through recombinant DNA technology that provides well established protocols for cloning, site directed mutagenesis and gene fusion in different host cells to allow the expression of specific proteins. These proteins can also be used to functionalize the polymers, that are aimed to be recognized by specific receptors found in cell membrane [90].

On the other hand, synthetic materials have the advantage of being produced with block units obtained of reliable source of raw material, and also be reproducible when manufactured in a large scale. They presented good mechanical properties and controlled degradation rate [75, 79, 87, 89, 91]. However, they have also disadvantages like the lack of biological functionality (i.e. do not present functional binding sites for cells) [79, 91], their degradation products can be toxic causing an adverse reactions if they are accumulated locally [89].

Another way to overcome the limitations of natural and synthetic polymers, is the merging of both polymeric blends. Polymeric material blends have good mechanical characteristics, easy processability and provide a control of the degradation rate of the system, to make the scaffold degradation rate match the growth rate of the regenerating tissue [91]. There are many polymeric blends between natural and synthetic polymers, such as alginate-acrylate, collagen-acrylate and starch-PCL [91-93]. An easy method to improve the mechanical properties of starch is by blending it with a suitable synthetic polymer. For example, a blend between starch and PCL has good mechanical properties and enzymatic degradation [94].

Although, synthetic and blends of biomaterials present an extensive range of properties, the natural biomaterials have a greater potential for attaining clinical success, considering their biocompatibility and role that some of these materials play in wound healing [95]. Some examples of natural, synthetic and blended polymers used in skin substitutes are presented in table 2.5.

Table 2.5: Natural, synthetic and composite materials used in the production of skin substitutes [91-93, 96].

Natural	Synthetic	Blends
Agarose	Cyclodextrin (CD)	Alginate/acrylate,
Alginic acid	Ethylene glycol (EG)	Alginate-g-(PEO-PPO-PEO)
Carrageenan	Hydroxyethyl methacrylate (HEMA)	Collagen-acrylate
Chitosan	Poly acrylamide (PAAm)	Hyaluronic acid-g-NIPAAm
Chondroitin	Poly acrylic acid (PAAc)	P(HEMA/Matrigel®)
Collagen	Poly acrylonitrile (PAN)	P(HPMA-g-peptide)
Dextran	Poly butylene oxide (PBO)	P(PEG-co-peptides)
Gelatin	Poly caprolactone (PCL)	P(PLGA-co-serine)
Hyaluronic acid	Poly ethyl methacrylate (PEMA)	Starch-PCL
Pectin	Poly ethylene glycol (PEG)	
Polylysine	Poly ethylene oxide (PEO)	
Starch	Poly glucosylethyl methacrylate (PGEMA)	
	Poly hydroxyl butyrate (PHB)	
	Poly hydroxypropyl methacrylamide (PHPMA)	
	Poly lactic acid (PLA)	
	Poly lactic-co-glycolic acid (PLGA)	
	Poly methyl methacrylate (PMMA)	
	Poly N-isopropyl acrylamide (PNIPAAm)	
	Poly N-vinyl pyrrolidone (PNVP)	
	Poly propyleneoxide (PPO)	
	Poly urethane (PU)	
	Poly vinyl acetate (PVAc)	
	Poly vinyl alcohol (PVA)	

2.4.3.1. Natural Polymers used in skin substitutes

Natural polymers are large macromolecules composed of a single or repeated monomer units, obtained from renewable resources such as plants, animals, and microorganisms. These polymers have high molecular weight, which influences their specific functions and structure [91].

Based on their composition, they can be divided into polysaccharides, proteins and nucleic acids. Most of these polymers are used in our diet and have structural similarity with the ECM components. Due to that, some of them have been used in a variety of human applications such as pharmaceutical excipients, drug delivery and skin substitutes [91, 97, 98].

Nowadays, with the advances of biotechnology, there is an increasing interest in using microorganisms to produce polymers by fermentation (enabling large-scale production,

avoiding complex and time-consuming isolation procedures, and the risk of transmission of animal-derived pathogens) [77]. Such allows the control of the polymer properties such as the molecular weight, branching patterns and branch chain lengths, and cross-linking between chains, constituting a key factor in Tissue Engineering [97, 99]. Although, the largest amount of polymers required for biomedical applications is still extracted from plant and animal sources or from algae [77]. Among them, polysaccharides are the most used in biomedical applications [98].

2.4.3.1.1. Polysaccharides

Polysaccharides are a group of polymers that can be formed with one type of monosaccharides or different (two or more - heteropolysaccharide) units joined together through glycosidic linkages. Many of them contain branched structures and are chemically modified by the addition of other molecules [91].
Differences in the monosaccharide structure, linkage types and patterns, chain shapes, and molecular weight, define their physical properties, including solubility, gelling potential and/or surface and interfacial properties[97]. Chitosan, dextran and agarose are examples of polysaccharides widely used for the production of skin substitutes.

- **Chitosan**

Chitosan is a linear heteropolysaccharide composed by ß-(1-4)-linked d-glucosamine and N-acetyl-d-glucosamine randomly distributed (see figure 2.9a). It is easily obtained by the full or partial alkaline deacetylation of chitin, obtained from crustaceans or insects exoskeleton and cell walls of most fungi and algae [77, 91, 100-103]. Chitosan is only soluble at low pH due to the protonation of amine groups under low pH conditions [104]. An important parameter to take into account on chitosan is the degree of N-deacetylation (% of d-glucosamine residues), this degree has a striking effect on solubility, reactivity, biodegradability and cell response [77, 91]. The chemical structure of chitosan allows the production of 2D or 3D structures with bactericidal activity, biodegradable, biocompatible, fungistatic, haemostatic, non-antigenic, non-carcinogenic properties, and with remarkable affinity to proteins. These properties make chitosan an excellent candidate to be use as scaffold in the biomedical field, namely for the wound healing process [80, 91, 102, 104, 105]. Sparkes and Murray developed a skin substitute made with chitosan and gelatin, that had excellent adhesion to subcutaneous fat in order to overcome the absence of adhesion of conventional biological dressings [106]. Hu et al., showed the importance of the presence of chitosan on bacterial activity and cell attachment [107]. Ishihara and collaborators created a chitosan hydrogel that promoted wound contraction and accelerated the wound closure and healing. Furthermore, chitosan was also shown to be an effective material for the delivery of GFs (eg. bFGF) directly involved on wound healing [108]. Recently, it was also demonstrated that chitosan can be used as a carrier for keratinocyte and fibroblast cells to the wound site in order to promote the healing process [109].

- **Dextran**

Dextran is a natural polymer that can be produced either chemically or by bacteria (eg. *Leuconostoc mesenteroides* and Streptococcus sp.) from sucrose. Structurally (figure 2.9b), dextran consists predominantly of linear α-1,6 linked d-glucopyranose residues that have three hydroxyl groups per anhydroglucose unit, which makes it very soluble in water [110-113]. The main properties of dextran are biocompatibility and biodegrability. This polymer is degraded by dextranases available in different organs of the human body (liver, spleen, kidney and colon) [112]. Due to these properties, dextran has benn used in the clinic for a long time. Initially it was used for plasma volume expansion, promotion of peripheral flow, and as an antithrombolytic agent [110]. Recently, dextran has also been used to produce hydrogels for biomedical applications, however it requires chemical modifications [114]. Being some examples of dextran based materials are glycidyl methacrylate dextran [115], hydroxyethyl methacrylate dextran [116], oligolactate-grafted dextran [117] and oxidized dextran [118].
Recent studies showed that oxidized dextran hydrogel may be used to enhance the wound healing process. Weng et al. developed an *in situ* gelable hydrogel composed of N-carboxyethyl chitosan and oxidized dextran that revealed to be versatile, non-toxic, *in situ* crosslinkable, biodegradable, capable to encapsulate cells and also enhance wound healing [119]. Kim and collaborators, showed the role of oxidized dextran to increase the hydrophilicity of silk fibroin, which is fundamental to create a moist environment at the wound site, for improving healing [120]. Moreover, oxidized dextran hydrogels have also been used as carriers of GFs to the wound site [121].

- **Agarose**

Agarose is a linear and neutral polysaccharide obtained from a marine algae (eg. Rhodophyta) that forms thermorreversible gels upon cooling agarose aqueous solutions. The chain of agarose is formed by disaccharide units of 1,3-linked-ß-d-galactopyranose and 1,4-linked-3,6-anhydro-α-l-galactopyranose (figure 2.9c) [91, 122-125]. The thermal and mechanical properties of agarose hydrogels are affected by polymer concentration, pH and solvent type [124]. The biocompatibility of agarose and its capacity to form very strong gels, even at low concentrations, make this polysaccharide suitable for applications in Tissue Engineering, however, agarose hydrogels exhibit a lower cell adhesiveness and slow degradation rate [123]. To overcome these limitations, agarose can be chemically functionalized or combined with other polysaccharides [123-125]. Gómez-Mascaraque et al. developed a hydrogel (chitosan/agarose) that allowed cell adhesion and proliferation [124]. Zhang and collaborators combined hyaluronic acid with agarose and produced a hydrogel that revealed a great potential to improve the wound healing [125].

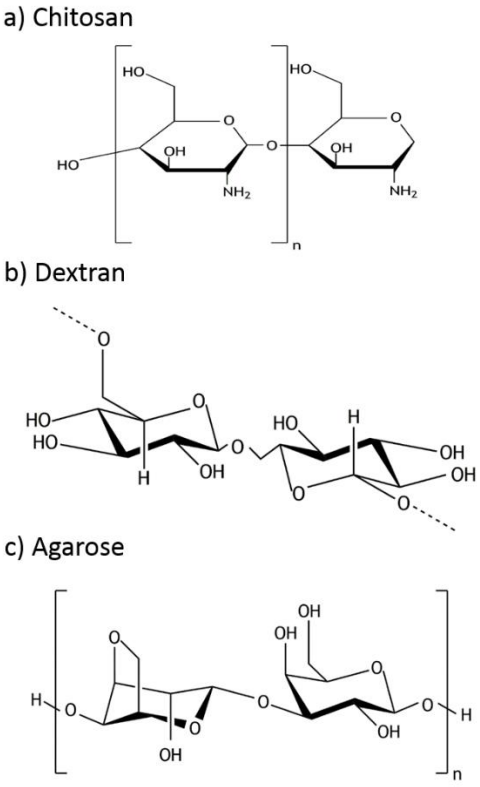


Figure 2.9: Chemical structure of Chitosan (a), Dextran (b) and Agarose (c).

2.4.3.2. Biomaterials generations

The biomaterials have suffered a continuous evolution along time. This evolution allows the division of biomaterials in four generations that differ in their complexity and capacity to adjust and/or interact with the living systems [126-130].

The first generation of biomaterials were not developed especially for medical applications but for being used as industrial high tech materials. For that, many of the selected materials showed to be either pathogenic and toxic [127, 129, 130]. Nowadays, and considering the toxicological aspects most of them are not used in biomedical applications. Although, some of them, like titanium alloy is still in use, since it does not have side effects [129, 130].

In order to overcome the problem of pathogenic and/or toxicity, a second generation of materials was developed. These materials were aimed to be used in biomedical field, for "achieve an appropriate combination of physical properties to match the tissue replaced with a minimal toxic host response" [126, 127]. These materials had as main features, durability and good mechanical properties, but were not degradable and bioinert (i.e., they show minimal effects *in vivo*) [130-132]. This second generation of materials are still in use today. Ceramics and metallic biomaterials are examples of materials of this second generation [130].

The third generation of biomaterials englobes bioactive materials could elicit a controlled action and reaction in physiological environment of the body [126, 127]. Besides being bioactive, they can be resorbable, with degradation rates that could be tailored to the

requirements of a desired application [127, 131]. In order to obtain these properties, some materials were chemically modified to have similar composition to those found in biological systems [126]. Chitosan hydrogel, described in chapter 3, is an example of the third generation of these materials [102].
Finally, the biomaterials of fourth generation are composites constituted by materials and biological components (eg. proteins and cells) [129, 130]. These biomaterials interact with the organism by different ways, such as, regulation of biological processes, real integration into the biological system, degradation of the materials in the biological system or by being involved in the fight against infection after its implantation. This generation include also, smart biomaterials, which have the ability to change their characteristics and performance depending on external stimuli (eg. temperature and pH), or may contain sensor functions to monitor their function and interaction with the biological systems [126]. Furthermore, they have also the ability to instruct/induce or trigger/stimulate effects on the cells and tissues [133]. Dextran hydrogel with growth factors encapsulated, described in chapter 4, is an example of the fourth generation of biomaterials [113].

Biomaterials have different applications in the field of Tissue Engineering. They are applied as space filling agents, delivery vehicles for bioactive molecules, and as 3D scaffolds that promote the cellular activity and can present stimuli to direct the formation of a desired tissue.

2.4.4. Skin Substitutes developed in the area of Tissue Engineering

The term tissue engineering was introduce by Yuan-Cheng Fung of the University of California, San Diego, in 1985 [134]. Tissue Engineering is an interdisciplinary field that integrates engineering and life sciences principles and methods to know the structure-function relationships in normal and pathological tissues. Tissue Engineering uses living cells, biocompatible materials and bioactive factors (eg. GFs) to maintain, repair, improve, and/or replace tissues and organs (see figure 2.10) [24, 135-137].
After an injury, the restoration of the outmost barrier is the most urgent step, i.e., coverage in order to avoid bacterial contamination, reduce water/blood loss and control pain [62]. However, traditional dressings cannot restore the structure and functions of the native skin, and problems such as fragility of the graft, wound contraction and scar formation often occur. New wound dressings have been produced based on the recent advances in the biomaterials field and also on cell culture. Such dressings may be, in a near future, effective therapeutic weapons for acute and chronic skin wounds [23, 67].

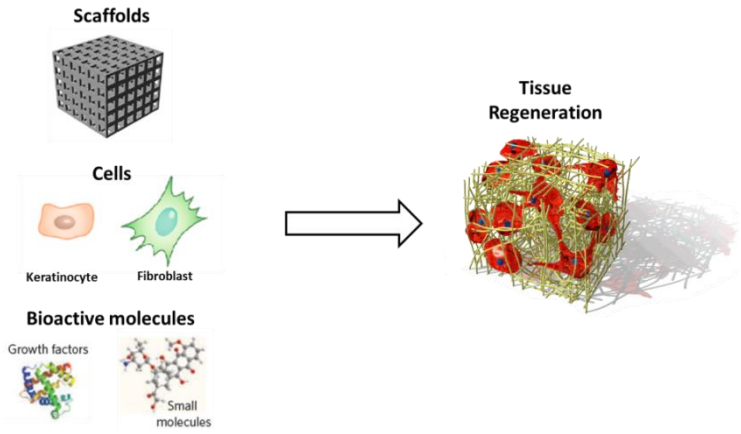


Figure 2.10: Scheme of the main components used in Tissue Engineering for the production of new tissues.

There are two principal approaches that have been used in the development of engineered tissues: the *in vitro* approach consists on the use of specific cells (eg. autologous cells), growing them on a biomimetic scaffold, under controlled culture conditions [138]. After obtaining a construct with adhered cells, it is delivered to the desire site in the patient's body [24, 139]; the *in vivo* approach uses an acellular biomimetic material for local tissue cell recruitment and also induces cell differentiation to form the required tissue.

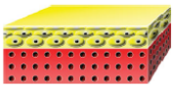
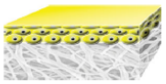
Nowadays several Tissue Engineering skin products are available in the market. The skin-substitute products can be organized according to anatomical structure, into epidermal (table 2.6) dermal (table 2.7) and dermo-epidermal (table 2.8) [140].

There are skin substitutes that only promote the replacement of the injured epidermis. The key step for the design and production of an epidermal substitute is to isolate the keratinocytes from a donor and to perform their subsequent *in vitro* culture, in order to obtain the necessary number of keratinocytes for the therapeutic purpose [140]. The use of autologous keratinocytes is advantageous since the risk of rejection is very low, however this kind of substitute presents some disadvantages, such as long culture time (about 3 weeks), difficulty to handle/apply and high production costs. In this way, several epidermal skin substitutes are already commercially available.

For example, Epicel® or Epibase® uses patient's own keratinocytes derived from a small skin biopsy, which are grown to confluency within 15 days to form cultured epithelial autografts sheets [141]. Laserskin® consists of autologous keratinocytes cultured on a hyaluronic acid membrane, which is laser-micro perforated. This allows the keratinocyte migration from a support material down to the wound bed [142]. On the other hand, CellSpray® uses either cultured or non-cultured autologous keratinocytes. This technology is based on a skin biopsy and a ready-made kit, thus surgeons can create a suspension of the skin's basal cells (the stem cells of the epidermis) and then spray the solution directly onto the burn, with results comparable to the other skin grafts. This allows the *in vivo* cell proliferation and differentiation and subsequently the wound closure to form a epithelial structure without forming a scar [143,

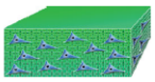
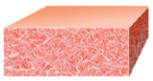
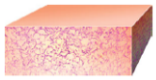
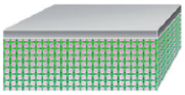
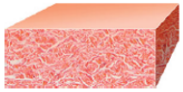
144]. These substitutes are being used to treat partial-thickness burns and chronic ulcers [145, 146].

Table 2.6: Epidermal skin substitutes available in the market to be used in the clinic [140, 147, 148].

Commercial Product	Description	Application
 **Epicel®** and **Epibase®**	Cultured autologous keratinocytes in polyurethane sheet	Dermal or full thickness burns
 **Epidex®**	Cultured autologous keratinocytes obtained from the outer root sheath of scalp hair follicles in silicone menbrane	Full- and partial-thickness burns and chronic ulcers treatment
 **Laserskin®** Or **Vivoderm®**	Autologous keratinocytes seeded on esterified laser-perforated hyaluronic acid membrane.	Deep partial- and full-thickness burns.
 **BioSeed-S®**	Subconfluent autologous keratinocytes on a fibrin matrix	Chronic venous leg ulcers treatment
 **Myskin®**	Cultured autologous keratinocytes seed on specially treated silicone sheet	Pressure and diabetic foot ulcers, superficial burns and skin graft donor sites.
 **CellSpray®**	Cultured autologous keratinocyte cell suspension spray	Partial-thickness and graft donnor site wounds

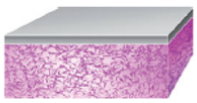
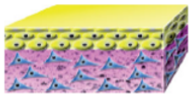
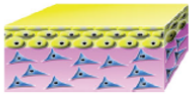
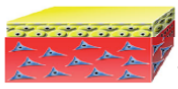
Dermal substitutes are generally acellular, based either on allogeneic, xenogeneic or synthetic materials [149]. Table 2.7 describes some of the dermal substitutes that are already commercially available [140]. Dermal skin replacements present advantages, such as reduced costs, easier manufacture and rigorous quality control. They also add mechanical stability and prevent the wound from contracting [24, 145]. However, they can be rejected and transmit diseases [150]. Alloderm® is a freeze-dried human acellular dermal matrix, with preserved basement membrane, acting similarly to a cadaver allograft. This type of matrix is ready to be incorporated into the wound, without any rejection and does not cause any immunogenic response due to the absence of a cellular component [151]. In turn, Biobrane® is a temporary dressing of partial-thickness burns and wounds, which consists on a pseudo-epidermal semipermeable silicon film bonded to nylon fabric with incorporated porcine collagen [152]. Hyalograft® 3D has no pseudo-epidermal layer but the product's effects are strengthened by the cultured autologous fibroblasts that provide the wound with GFs and cytokines. This material is reported to improve *in vitro* epithelial organization and dermal- epidermal junction maturation in organotypic skin bioconstructs [153].

Table 2.7: Dermal skin substitutes available in the market to be used in the clinic [140, 147, 148].

Commercial Product	Description	Application
 Dermagraft®	Cultured allogeneic nenonatal fibroblasts on a biodegradable polyglactin mesh	Full-thickness burns and chronic wounds
 Alloderm®	Acellular dermal matrix from human allograft	Full- and partial-thickness wounds treatment
 EZ-Derm®	Aldehyde-crosslinked porcine dermal collagen	Full- and partial-thickness wounds treatment
 Biobrane®	Porcine collagen chemically bound to silicone/nylon membrane	Temporary covering of superficial and partial-thickness burns and wounds
 Hyalograft 3D®	Esterified hyaluronic acid matrix seeded with autologous fibroblasts	Full- and partial-thickness wounds treatment
 Matriderm®	Bovine dermal collagen type I, III, V and elastin	Full- or partial-thickness wounds treatment

Composite skin substitutes or Dermo-epidermal skin substitutes aim to mimic the native structure of normal skin, where both epidermal and dermal layers are presented [154]. These substitutes are more advanced and sophisticated than the epidermal and dermal ones, but also the most expensive. The current commercially available dermo-epidermal (composite) skin substitutes are listed in table 2.8.

Table 2.8: Dermo-epidermal skin substitutes available in the market to be used in the clinic [140, 147, 148].

Commercial Product	Description	Application
 Integra®	Thin polysiloxane (silicone) layer; cross-linked bovine tendon collagen type I and shark glycosaminoglycan (chondroitin-6-sulfate)	Full- or partial-thickness wounds treatment
 OrCel®	Human allogeneic skin cells (fibroblasts and keratinocytes) are cultured in two separate layers into a type I bovine collagen sponge.	Treat skin graft donor sites and mitten-hand surgery for epidermolysis bullosa
 Apligraf®	Human neonatal skin cells (fibroblasts and keratinocytes) are cultured in two separate layers into a type I bovine collagen lattice.	Treat chronic venous leg ulcers and diabetic foot ulcers.
 TissueTech® autograft system	Combination of Hyalograft 3D® and Laserskin®	Full- and partial-thickness burns and chronic ulcers treatment

Apligraf® has on its composition viable allogeneic neonatal fibroblasts grown in a bovine collagen type I gel matrix, combined with viable allogeneic neonatal keratinocytes, forming a confluent superficial layer on the construct, thus mimicking the normal structure of human skin [155, 156]. Orcel® is a tissue-engineered skin construct that includes cultured allogeneic fibroblasts and keratinocytes, obtained from the same neonatal foreskin. Fibroblasts are seeded into a bovine type I collagen sponge, which has a non-porous collagen-gel coating, on top of which keratinocytes are seeded to form a confluent layer [140]. These composite substitutes can be used to treat skin graft donor sites and venous and diabetic foot ulcers [146].

Despite the existence of these skin constructs, none of them is able to completely replicate the anatomy, physiology, biological stability or aesthetic nature of the native skin [11, 157-159]. Their clinical implementation remains riddled with flaws, such as poor preservation and/or restoration of body image (cosmesis) and also a high rate of infections associated with their application. Furthermore, these approaches are quite expensive, requiring frequent dressings change and making the patient more susceptible to subsequent secondary bacterial infections [160]. Bearing this knowledge in mind, there is a huge demand in the development of alternative strategies for the treatment of burns and other types of skin injuries. In the last decade, there has been an increasing interest on the Tissue Engineering approach of dermal and epidermal layers using natural or synthetic matrices [161].
The success of skin substitutes is dependent on finding an appropriate material that create a microenvironment that mimics the cellular and tissue complexity found *in vivo* by incorporating physical and chemical signals within engineered 3D scaffolds. Hydrogels are ideal materials to be used as 3D tissue scaffolds, once they mimic the ECM.

2.4.4.1. Hydrogels used in the area of Tissue Engineering

Hydrogels are hydrophilic 3D networks that are able to absorb significant amounts of water or biological fluids, while remaining insoluble in aqueous solutions [92, 96, 162-165]. Their capacity of absorb fluids is attributed to the presence of hydrophilic groups such as –OH, –CONH–, $–CONH_2–$, and $–SO_3H$ in the polymers used for production of hydrogels [92, 166]. The high water content of hydrogels, confer them a similar structure to that found in ECM [164, 167], acting as artificial ECMs, that have an important role on the cellular recognition.
Hydrogels, in Tissue Engineering, must meet a number of criteria in order to promote the formation of new tissue. These criteria include the classical parameters such as the biocompatibility and physical (eg, degradation and mechanics) and biological (eg, cell adhesion) properties. Furthermore, they are suitable for the cleaning of dry, sloughy or necrotic wounds by rehydrating dead tissues (moist healing), increase autolytic debridement and cool the surface of the wound, which may lead to a marked reduction of pain and consequently increase the patient acceptability. Hydrogels, due to their chemical properties, do not adhere to the wound and can be easily removed, if it is required. Those hydrogels that fulfill all these properties can be used as 'ideal dressing' [23, 93, 168].

The polymers normally used in the synthesis of hydrogels can have natural or synthetic origins. However, natural polymers such as agarose, alginate, chitosan, collagen and dextran have a clear advantage due to their biocompatibility.

Examples of commercially available hydrogels for skin regeneration are: AquaDerm™ Hydrogel Sheet (DermaRite), Dermagel™ Hydrogel Sheets (Medline Industries), Tegaderm™ Hydrogel Wound Filler (3M).

Hydrogels can be classified based on a variety of characteristics such as, neutral or ionic (based on the nature of the side groups), affine or phantom networks (according to their mechanical and structural features), homo- or co-polymers network (based on the method of preparation), amorphous, semicrystalline, hydrogen bonded, supermolecular, and hydrocollodial (based on the physical structure) [92, 164]. Moreover, they can also be classified based on their responsiveness to the physiologic environment stimuli (pH, ionic strength, temperature, electromagnetic radiation, etc) [92, 164].

Hydrogels can also be used as drug delivery systems, combining the previously described properties with controlled release of bioactive molecules into the wound bed [169]. However, in the majority of the cases it is desired to have a slow release profile of the bioactive molecules to ensure its presence during the entire period of healing. In order to attain such objective, some researchers have incorporated GFs into the artificial ECM. These GFs, are determinant in the wound healing process (previously described in table 2.1), and are released along the time. They are recognized by cells reach the interior of the scaffold [170]. Liu et al. showed the potential of glycosaminoglycan hydrogel on topical delivery of FGF, in patients with compromised wound healing [171]. To enhance the recognition of ECM by cells and to promote the cell adhesion, RGD sequences similar to natural ECM proteins have been incorporated on hydrogels [172].

Hydrogels appeared as a good candidate for protein/drug release due to their properties, since they are able to maintain protein structure and drug activity. However, controlled release of protein/drug over a long time period is not possible using hydrogels. The rate of protein/drug release from this 3D matrix is generally diffusion-controlled through aqueous channels within hydrogels [173]. To overcome this limitation, different strategies have been developed, like the incorporation of microparticles as can be observed in figure 2.11 [174].

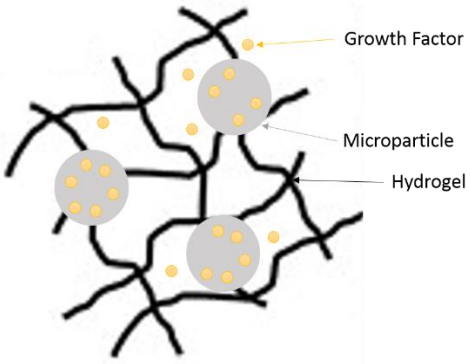


Figure 2.11: Illustration of microparticles loaded with growth factors, encapsulated in hydrogels.

Liposomes, microparticles and nanoparticles have been developed and tested as drug delivery systems that enable a controlled drug release. Therefore, the incorporation of these carries within hydrogels may be fundamental to avoid a burst release from hydrogel of bioactive molecules. The entrapment of these systems within the network, increases the efficiency of bioactive molecules release in the targeted site *in vivo* [175]. Furthermore, the hydrogel acts as a support for the carriers incorporated in its polymeric matrix, assures the stability of drugs/molecules, and can also provide an extra diffusion barrier for drug release (increase the period along which drugs are released). Besides, it also allows the reduction of the number of therapeutic doses administered to the patient [113, 175]. Bae et al. designed a sustained-release system for local drug delivery that incorporated PLGA microparticles loaded with a model drug (5-fluorouracil) in a thermosensitive chitosan hydrogel. A decrease in the drug burst release was observed and so they proposed this system to be used as an injectable drug carrier [176].

Taking into account the excellent properties of hydrogels and the importance of sustained applications of bioactive molecules (eg. growth factor) in wound healing, these systems will be further studied for its future application in clinic.

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Chapter 3
Paper I

Wound Repair and Regeneration

Development of a new chitosan hydrogel for wound dressing

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3.1. Abstract

Wound healing is a complex process involving an integrated response by many different cell types and growth factors in order to achieve rapid restoration of skin architecture and function. The present study evaluated the applicability of a chitosan hydrogel (CH) as wound dressing. Scanning electron microscopy analysis was used to characterize CH morphology. Fibroblast cells isolated from rat skin were used to assess the cytotoxicity of the hydrogel. CH was able to promote cell adhesion and proliferation. Cell viability studies showed that the hydrogel and its degradation by-products are non-cytotoxic. The evaluation of the applicability of CH in the treatment of dermal burns in Wistar rats was performed by induction of full-thickness transcutaneous dermal wounds. Wound healing was monitored through macroscopic and histological analysis. From macroscopic analysis, the wound beds of the animals treated with CH were considerably smaller than those of the controls. Histological analysis revealed lack of reactive or granulomatous inflammatory reaction in skin lesions with CH and the absence of pathological abnormalities in the organs obtained by necropsy, which supported the local and systemic histocompatibility of the biomaterial. The present results suggest that this biomaterial may aid the re-establishment of skin architecture.

Keywords: Biocompatibility; Biodegradable; Cell adhesion; Chitosan Hydrogel; Wound healing

3.2. Introduction

Skin lesions are traumatic events that lead to the increase of fluid loss, hypothermia, scarring, locally immunocompromised regions, infections and a change of body image [1]. Despite advances in therapy, infections remain a leading cause of morbidity and mortality in burn patients [2].

After skin damage, wound healing is a complex biological process, which includes a wide range of mechanisms, such as coagulation, inflammation, matrix synthesis and deposition, angiogenesis, fibroplasia, epithelisation, contraction and remodelling [1, 3]. In spite of its complexity, regeneration of skin is often imperfect and the wound is mainly covered by scar tissue [4].

The replacement of damaged tissues requires biocompatible materials on which cells may adhere and proliferate. Such materials include natural polymers extracted from the native extracellular matrix (ECM), like collagens and glycosaminoglycans [4]. However, some of these materials, due to their chemical and biological inertness may be unable to induce cell adhesion and proliferation. It is well known that adhesion and proliferation of cells to biomaterials is highly dependent on the topography of the substratum and its surface properties, namely its surface charge, surface free energy and density, along with the nature of its polar groups [5, 6].

In past decades, many skin substitutes such as xenograft, allografts, and autografts have been used for the treatment of deep partial and full thickness wounds. However, due to the antigenicity or the limitation of donor sites, skin substitutes cannot accomplish the purpose of skin regeneration and hence are not widely used [7].

Nowadays, none of the skin substitutes available or under development are able to fully substitute natural living skin [8].

Chitosan is the deacetylated derivative of chitin, a natural polysaccharide found primarily in the exoskeletons of arthropods and some fungi [9]. It is a linear polysaccharide comprising copolymers of glucosamine and *N*-acetyl glucosamine linked by ß (1-4) glycosidic bonds. The molar fraction of glucosamine residues is referred to as the degree of deacetylation (DD) [10, 11]. In its crystalline form, chitosan is normally insoluble in aqueous solution above pH 7; however, in diluted acids (pH 6.0), the protonated free amino groups on glucosamine facilitate solubility of the molecule. Chitosan preparations of various molecular weights (50-2000kDa), degrees of deacetylation (30-95%) and further molecular derivatization patterns, allow extensive adjustment of mechanical and biological properties. These properties include anticholesterolemic and antimicrobial activity, biocompatibility, biodegradability, fungistatic, hemostasis, non-carcinogenic, remarkable affinity to proteins, stimulation of healing, tissue engineering scaffolds, and drug delivery [2, 3, 12-15].

Recently, there has been a growing interest in the chemical modification of chitosan in order to improve its solubility and widen its applications [16]. Different studies have reported the use of chitosan for skin tissue engineering [13]. Ueno et al. [17] showed that chitosan in the

form of chitosan-cotton, accelerates wound healing by promoting infiltration of polymorphonuclear cells at the wound site. In recent studies, chitosan has been used to deliver bioactive molecules: basic fibroblast growth factor[18] and human epidermal growth factor [12] were encapsulated in this biomaterial; electrospun nonwoven nanofibrous hybrid mats based on chitosan and poly[($_{L}$-lactide)-co-($_{D,L}$-lactide)] were produced [19]; chitosan dressing incorporating a procoagulant (polyphosphate) and an antimicrobial (silver) [15]; chitosan acetate bandages were used as a topical antimicrobial dressing for infected burns [2].

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3.3. Materials and Methods

3.3.1. Hydrogel synthesis

Chitosan (average M_{η}= 270 000 Da and deacetylation degree 86%) was obtained from Cognis, (Monheim am Rhein, Germany). Lactic acid (> 99,0%) was purchased from HiMedia, (Mumbai, India). Ammonium hydroxide solution at 25% (puriss. p.a.) was acquired from Fluka, (Buch, Switzerland).

The Chitosan hydrogel (CH) was produced adapting the method previously described by Montembault et al. [11] Briefly, to prepare the CH, a chitosan solution 4% (w/w) was dispersed in lactic acid 2% (v/v) to achieve the stoichiometric protonation of the $-NH_2$ sites, followed by agitation until complete dissolution. The solution was left overnight in order for the air bubbles to collapse completely. Chitosan solution was poured into several small moulds (14x7cm), 30g per mould. The moulds were placed inside a hermetic chamber, together with 4L of ammonia solution, 2,5% (v/v). The chitosan solutions were left exposed to ammonia vapour overnight. The hydrogels were rinsed with distilled water, removed from the moulds and placed in watch glasses for 5 hours in order for the excess ammonia to evaporate. The prepared CH was packed separately and sealed in plastic bags. The samples were labelled and sterilized by UV radiation for 30 minutes. The packed sterilized CH was then maintained at room temperature in a dry and clean place until use.

3.3.2. Scanning electron microscopy

The morphologies of CH with/without adhered fibroblasts cells isolated from rat skin were characterized by scanning electron microscopy (SEM). CH and their adherent fibroblasts were fixed overnight with 2.5% glutaraldehyde in phosphate-buffered saline (PBS) at 4 ºC. Samples were rinsed three times with PBS buffer for 2 minutes and dehydrated in graded ethanol (ETOH) of 70%, 80%, 90% and 100%, 5 minutes each. Then, hydrogels were mounted on an aluminium board using double-sided adhesive tape and sputter coated with gold using an Emitech K550 (London, England) sputter coater. The samples were analysed using a Hitachi S-2700 (Tokyo,

Japan) scanning electron microscope operated at an accelerating voltage of 20 kV at various magnifications.

3.3.3. Cell source and growth

Fibroblast cells from rat skin were obtained as reported previously [20]. The operative skin area was shaved and disinfected using 70% ETOH. Skin samples were aseptically removed from the rats and stored in RPMI-1640 (Gibco, Grand Island, NY) medium with penicillin G (100 U/ml), streptomycin (100 µg/ml) and amphotericin B (0.25 µg/ml). Then samples were minced and incubated for 3 hours in 0.1% collagenase solution (37 °C, 5% CO_2). After incubation, the samples were centrifuged (5 minutes, 250× g), the supernatant was discarded, and the pellet was washed with Dulbecco's modified Eagle's medium (DMEM)-F12 supplemented with heat-inactivated fetal bovine serum (FBS, 10% v/v). The isolated cells were plated in 25 cm^3 T-flasks with DMEM-F12 medium (1:1 v/v) supplemented with heat-inactivated FBS (10% v/v), $_L$-glutamine (2 mM), penicillin G (100 U/mL), streptomycin (100 µg/mL) and amphotericin B (0.25 µg/ml).
After 2 hours, the non adherent cells were washed out. Cells were kept in culture at 37 °C, in a humidified atmosphere, 5% CO_2. After confluence was attained, cells were sub-cultivated by a 5-minute incubation in 0.18% trypsin (1:250) and 5 mM EDTA. The free cells were added to an equal volume of culture medium. Following centrifugation, cells were resuspended in sufficient culture medium.

3.3.4. Proliferation of fibroblast cells in the presence of CH

To examine cell proliferation, fibroblast cells were cultured in 24-well plates at $1x10^5$ cells/ml, for 24 hours. Cell growth was monitored using an Olympus CX41 (Tokyo, Japan) inverted light microscope equipped with an Olympus SP-500 UZ digital camera and SEM images were also acquired

3.3.5. Determination of hydrogel cytotoxicity by 3-[4,5-dimethyl-tiazol-2-yl]-2,5-diphenil tretrazolium bromide (MTT) assay

CH was applied to a 96-well plate (Nunc, Roskilde, Denmark). The plates were UV irradiated for 30 minutes, before cell seeding.
Second passage rat fibroblasts cells were seeded in a 96-well plate containing the biomaterial at a density of $6x10^4$ cells per well. Then, 100 µl of culture medium was added to each well and the plate was incubated at 37 °C, in a 5% CO_2 humidified atmosphere, for 24 hours. After incubation, the mitochondrial redox activity was assessed through the reduction of the MTT, (n=6). Fifty µl of MTT (5 mg/ml PBS) was added to each sample, followed by incubation for 4 hours at 37 °C, in a 5% CO_2 atmosphere. The medium was aspirated and cells were treated with

50 µl of isopropanol/HCl (0.04 N) for 90 minutes. Absorbance at 570 nm was measured using a Biorad Microplate Reader Benchmark (Tokyo Japan).
Wells containing cells in culture medium without biomaterials were used as negative control. ETOH 96% was added to wells containing cells as a positive control.

3.3.6. Animal experiments

A total of 18 female Wistar rats (8-10 weeks) were used, weighting between 200 and 250 g at the time of the experiments. The animal protocol followed in the present study were approved by the Ethics Committee of Centro Hospitalar Cova da Beira and were performed according to the guidelines set forth in the National Institutes of Health Guide for the care and use of laboratory animals.
Rats were individually anesthetized via an IP injection (40 mg/kg ketamine, 5 mg/kg xylazine) for surgery and induction of the burn wound. The operative skin area was shaved and disinfected using ETOH. Then, the dorsal skin of the animal was exposed to water at 95 ± 1 °C for 10 seconds. After 2 hours, damaged tissue was removed with surgical scissors and forceps. Wounds with 2 cm diameter were created with no visible bleeding. The animals were divided into two groups: in group 1, wounds were filled with CH and finally fixed with elastic bandage; group 2 was used as control and wounds were covered with PBS and an elastic bandage.
After surgery, animals were kept in separate cages and were fed with commercial rat food and water *ad libitum*. All animals showed good general health condition throughout the study, as assessed by their weight gain. The animals were sacrificed 7, 14 and 21 days after.

3.3.7. Histological study

The material from the skin lesions and organs (brain, heart, lung, liver, spleen and kidney) obtained by necropsy was formalin fixed and paraffin embedded for routine histological processing. A 3 µm section obtained from each paraffin block was stained with hematoxylin & eosin (H&E) and evaluated in a blinded manner by two observers using a light microscope with specific image analysis software from Olympus. For the morphological evaluation of skin lesions, three parameters were considered: wound bed length, thickness of the granulation tissue layer, and thickness of the epithelial layer. In the assessment of the three parameters it was always considered the greatest dimension observed. Skin fragments with no CH were used as normal control. The assessment of brain, heart, liver, lung, kidney and spleen was performed by looking for any morphological alteration.

3.3.8. Evaluation of the wound size

Images of the wound area were taken by digital camera (Nikon D50, Ayuthaya, Thailand) and analyzed with image analysis software Image J (Scion Corp., Fredrick, MD). Measurement of

wound closure area was defined by the limits of grossly evident epithelisation, with all surface areas in a two-dimensional plane calibrated against the adjacent metric ruler. The percentage of wound size was calculated using the following formula: D_N/D_0 X 100 (%), where D_0 is the dimension of the full thickness circular skin wound area (2 cm diameter) on day 0, and D_N is the dimension of the wound area on the indicated day.

3.3.9. Statistical analysis

In each measurement of the surface area of the burn wounds, a minimum of three animals were used. The results obtained were expressed as mean ± standard error of the mean. Differences between groups were tested by one-way ANOVA with Dunnett's post-hoc test. Computations were performed using a MYSTAT 12 statistical package (Systat Software, a subsidiary of Cranes Software International Ltd.)

3.4 Results

3.4.1 Morphology of CH

From gross observation, the CH was white, opaque, and presented a dense outer layer, as shown in figure 3.1A. The dense outer layer of hydrogel provides several functions such as control of the water loss through evaporation and protection from external contamination, as previously reported in the literature [12].

SEM analysis (figure 3.1B-D) revealed a highly porous and interconnected interior structure. It could be inferred that the hydrogel has a high water retention capacity because high DD chitosan (86%) can establish H-bonds with water. Both small and macromolecules could freely diffuse into CH

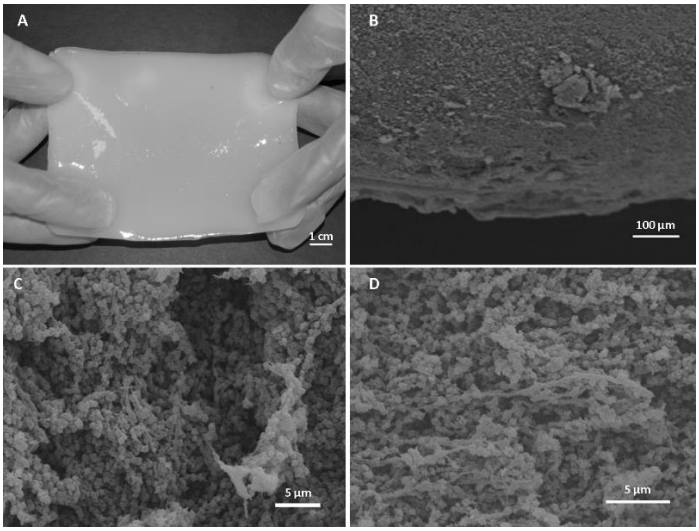

Figure 3.1: Photograph of chitosan hydrogel (CH) (A) and scanning electron microscopic images of CH surface morphology (B–D). Original magnification: (A) scale bar 1 cm; (B) X50, scale bar 100 µm; (C) X 2,000, scale bar 5 µm; (D) X3,000, scale bar 5 µm.

3.4.2 Cytotoxicity of CH

In order to study the applicability of our new hydrogel for biomedical applications, the cytocompatibility of CH was first studied through *in vitro* studies. Fibroblasts were seeded at the same initial density in the 96 well plates, with or without hydrogel, on day 0, to assess the CH cytoxicity. After 24 hours, cell adhesion and proliferation was visualized using an inverted light microscope (figure 3.2). Fibroblast cells adhere and grow in the vicinity of CH (figure 3.2A and B) and in the negative control (figure 3.2C). In the positive control, no cell adhesion or proliferation was observed. Dead cells with their typical spherical shape can be observed in figure 3.2D.

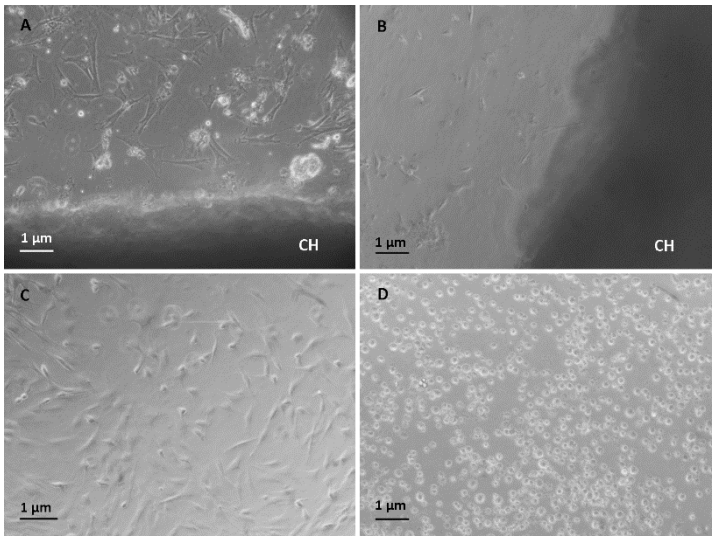

Figure 3.2: Photomicrographs of fibroblast cells from rat skin after being seeded on chitosan hydrogel (CH) after 24 hours (A) and 3 days (B); polystyrene (C); and polystyrene with ethanol 96% (D). Original magnification X100, scale bar 1 µm.

SEM images were acquired to further examine and characterize cell adhesion to CH. Cell growth and filopodia were observed, indicating that cells were attached and spread on hydrogels after 24 hours (figure 3.3A) and 7 days (figure 3.3B). Because of their large volume, the fibroblasts could not penetrate into the pore cavity and remained on the surface of CH. Fibroblasts synthesize and organize an ECM, which is fundamental for the repair of the lesion and avoid formation of hypertrophic scars and keloids [4].

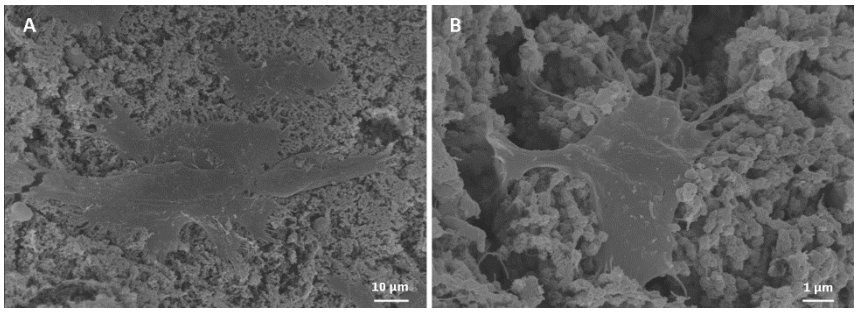

Figure 3.3: Scanning electron photomicrographs of fibroblasts adhered on the surface of chitosan hydrogel Original magnification: (A) X1,000, scale bar 10 mm; (B) X4,500 scale bar 1 µm.

To further evaluate the biocompatibility of the CH, MTT assay was also performed (figure 3.4). The MTT assay showed a significant difference between cells exposed to CH and the positive control ($p < 0.05$) after 24 hours of incubation, suggesting that the hydrogel did not affect cell viability. These results show that the tested formulation does not have an acute cytotoxic effect.

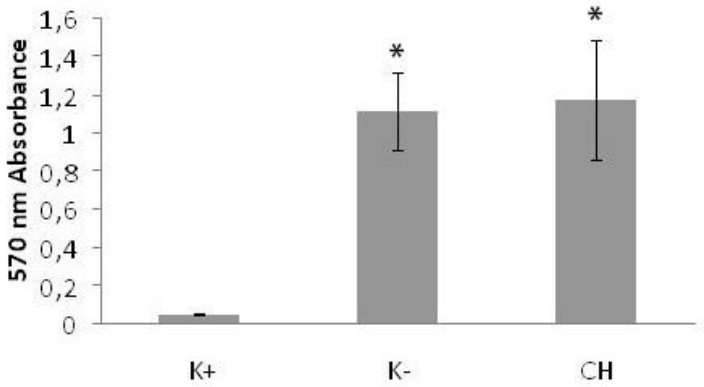

Figure 3.4: Cellular activities measured by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay. K+, positive control; K-, negative control; CH, chitosan hydrogel. Fibroblast cells in the presence of biomaterial. Each result is the mean ± standard error of the mean of at least three independent experiments. Statistical analysis was performed using one-way ANOVA with Dunnett's post hoc test (*p < 0.05).

3.4.3 Acceleration of wound healing by CH in rats transcutaneous full-thickness dermal wounds

In vivo experiments showed that CH adhered uniformly to the freshly excised wound surface, as previously reported in the literature [21]. In the inflammatory phase, chitosan has unique hemostatic properties that are independent of the normal coagulation cascade [3, 22].

Figure 3.5 shows a set of typical wound beds shortly after the surgical procedure and application of the hydrogel. The healing patterns were observed after 2, 6, 9, 13, 15 and 21 days and showed that the topical application of chitosan improved wound healing. The wound area decreased rapidly in the presence of hydrogel when compared with the control (figure 3.5 and 6). The results obtained were statistically significant until day 9.

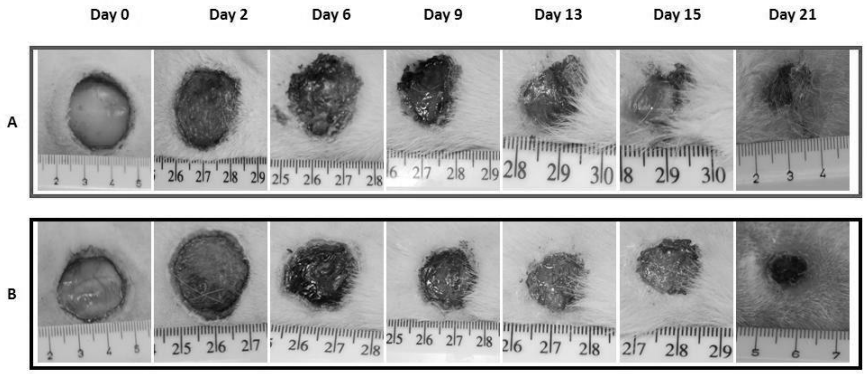

Figure 3.5: Typical macroscopic wound healing panorama with different treatments over 21 days. One deep third degree burn wound with 2 cm diameter at the dorsal skin of female Wistar rats, treated with chitosan hydrogel (A) and phosphate-buffered saline. (B). All went through various healing phases such as inflammation, eschar, tissue formation and tissue remodeling on the 2nd, 6th, 9th, 13th, 15th, and 21st day after injury.

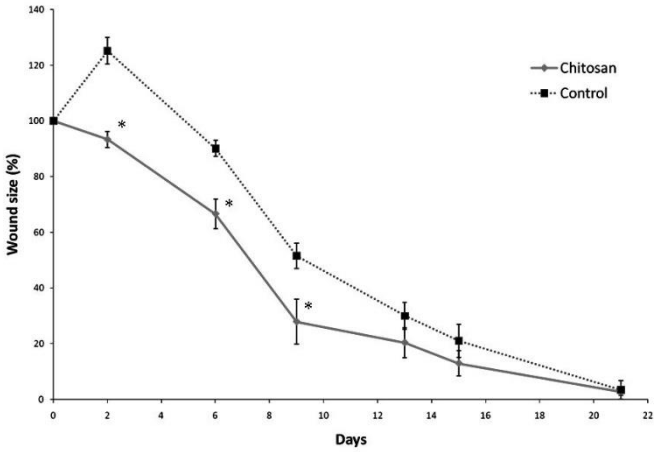

Figure 3.6: Effect of chitosan hydrogel and phosphate-buffered saline on burn wound. The surface area of the burn wounds was calculated as described in methods and reported at each time point as the percentage of the surface area at baseline. Each point represents the mean ± standard error of the mean of at least three independent experiments. *Chitosan vs. control ($p < 0.05$, one-way ANOVA with Dunnett's post-hoc test).

3.4.4 Histological study

The results of the histological study are summarised in figure 3.7. The analysis of histological data (figure 3.8) showed that the maximum and minimum values for wound bed length and granulation tissue thickness were obtained on day 14 and 21, respectively. Epithelial layer thickness increased progressively from day 7 to day 21. On day 21, all the skin lesions exhibited complete epithelisation. Neither specific inflammation nor reactive granulomas to the presence of CH were observed. No microorganisms were observed in skin lesions. No pathological abnormalities were observed in brain, heart, liver, lung, kidney and spleen obtained during necropsy.

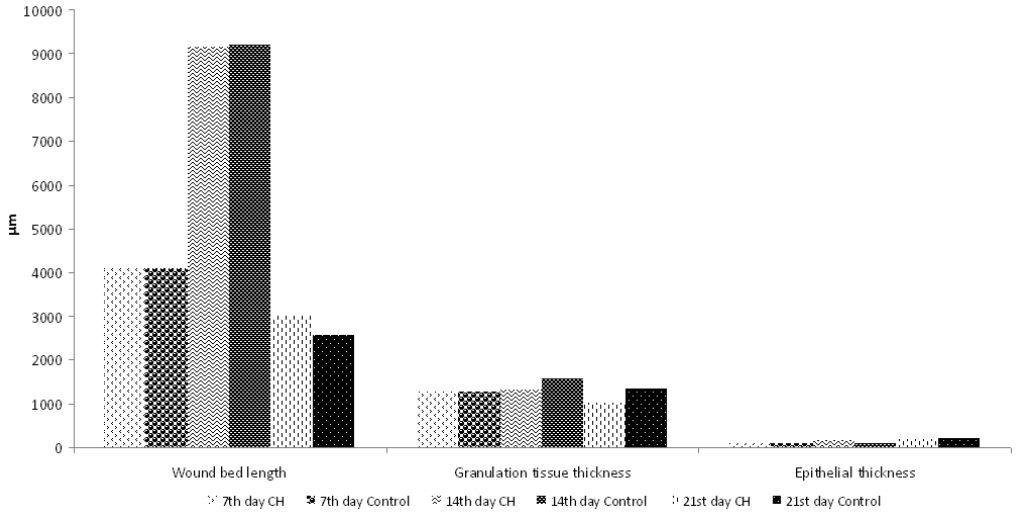

Figure 3.7: Graph with results of the histological analysis. For the morphological evaluation of skin lesions, three parameters were considered: wound bed length, thickness of the granulation tissue layer, and thickness of the epithelial layer. Skin fragments with no chitosan hydrogel (CH) were used as control.

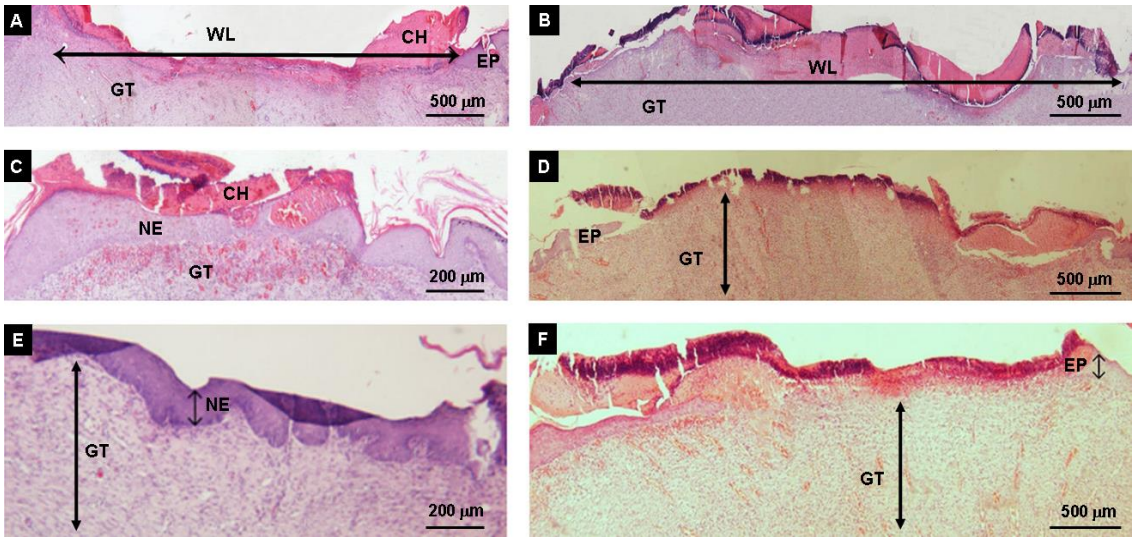

Figure 3.8: Hematoxylin and eosin stained sections of biopsies for the morphological evaluation of skin lesions. CH-treated wound on the 7th day, scale bar 500 µm (A), at 14th day, scale bar 200 µm (C), and at 21st day, scale bar 200 µm (E). Control wound on the 7th day (B), on the 14th day (D), and on the 21st day (F), scale bar 500 µm. CH, chitosan hydrogel, EP, epithelial layer, GT, granulation tissue, NE, new epithelial layer; WL, wound length.

3.5 Discussion

Wound healing is a dynamic process that typically evolves from its initial inflammatory response to complete resolution and thus, healing [23]. Hydrogels, with their high water contents and retention capacity, appear to be optimal media to enhance wound healing [23, 24], and thus, much interest has been focused on developing hydrogel-based wound dressings from biomaterials [23, 25].

In the present work, a CH was prepared through a recent development of the method previously described in the literature [11]. Chitosan is considered as an appropriate functional material for biomedical applications because of high biocompatibility, biodegradability, non-antigenicity and adsorption properties. Anti-inflammatory or allergic reactions have not been observed in human subjects following topical application, implantation, injection and ingestion [26]. In previous studies, Montembault et al. [11] reported that the percentage of DD and polymer concentration influences the mechanism of gelation of CH. For high DD, the high charge density is responsible for strong electrostatic repulsions, which disfavours the formation of physical junctions between chain segments. The present hydrogel was mainly built by hydrogen bonding. In high concentrated polymer solutions, chains become entangled, and hydrogel forms rapidly [27]. Lactic acid was added to induce the stoichiometric protonation of the $-NH_2$ sites.

Keeping in mind the wound dressing application, the porous section of CH (figure 3.1) promotes drainage, prevents the build-up of exudates, and may be an optimum wound bed for autografting. Moreover, the porosity of CH promotes gas exchange, which is fundamental for the wound healing process. A high CO_2 pressure increases the acidity and slows down the healing process, and in addition, a low oxygen concentration decreases the regeneration of tissue cell

or facilitates the proliferation of anaerobic bacteria [22]. Our CH appears to be particularly interesting for the proposed biomedical application, because it behaves as a decoy of biological media, both do to its physical form and by its chemical structure. Indeed, the β (1-4) glycosidic linkage and the N-acetyl groups are present in the structure of extra-cellular matrixes [27].

The observation of cell growth in the presence of CH (figures 3.2 and 3.3) revealed the importance of the method used to obtain the present hydrogel, because previous studies reported that there is no evidence that chitosan [28] or chitosan coated membranes [4] can support adhesion and proliferation of fibroblasts *in vitro*. It is well known that the surface chemistry of hydrogels can affect cell adhesion, proliferation and other phenomena [6]. Chitosan used to produce CH presents a DD of 86%, which is responsible for CH being positively charged at the surface. Hamilton et al. reported that increased *N*-acetylation of chitosan changes the physical properties of chitosan, as it becomes less positively charged and more hydrophobic [29]. The high DD of CH allows electrostatic interactions of cationic NH_3^+ groups with anionic glycosaminoglycans, proteoglycans and other negatively charged molecules that are present in the cell membranes [30]. Because skin has a negative charge and CH is a cation polymer, it can bind electrostatically to skin, as reported previously in the literature [31]. These electrostatic interactions are fundamental for the suitability of the material for the function propose.

In vitro and *in vivo* cytocompatibility studies revealed that CH and its degradation by-products are biocompatible, suggesting that CH has no cytotoxic effect.

From macroscopic analysis, the wound beds of the animals treated with CH were considerably smaller as compared to those of the controls treated with PBS. Macroscopic findings did not reveal significant difference in terms of wound contraction area after day 9.

The wound area of the control animals increased during the first days (figure 3.6), which was not observed in the animals treated with CH. Therefore, this support the promoting role of CH in wound healing. These results corroborate what has been reported previously in the literature [32-34].

In the histological study, the lack of reactive or granulomatous inflammatory reaction in skin lesions with CH and the absence of pathological abnormalities in the organs obtained by necropsy, supported the local and systemic histocompatibility of the biomaterial. Furthermore, the increasing thickness of the epithelial layer during the experiment and the presence of complete epithelisation in all the skin samples treated by CH, suggests that this biomaterial may aid the re-establishment of tissue architecture. In addition, the absence of microorganisms in skin lesions, after CH treatment, supports the previous described antimicrobial properties of CH [2] and supports its role in skin repair.

Further studies will be required to clarify the clinical significance of these findings for wound healing. The addition of grafted dermal fibroblasts to this natural polymer may help the remodelling of wounds and their perfect healing, as demonstrated for a number of skin substitutes.

3.6 Acknowledgements

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Chapter 4
Paper II

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Dextran based-hydrogel containing chitosan microparticles loaded with growth factors to be used in wound healing

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4.1. Abstract

Skin injuries are traumatic events, which are seldom accompanied by complete structural and functional restoration of the original tissue. Different strategies have been developed in order to make the wound healing process faster and less painful. In the present study *in vitro* and *in vivo* assays were carried out to evaluate the applicability of a dextran hydrogel loaded with chitosan microparticles containing epidermal and vascular endothelial growth factors, for the improvement of the wound healing process. The carriers' morphology was characterized by scanning electron microscopy. Their cytotoxicity profile and degradation by-products were evaluated through *in vitro* assays. *In vivo* experiments were also performed to evaluate their applicability for the treatment of skin burns. The wound healing process was monitored through macroscopic and histological analysis. The macroscopic analysis showed that the period for wound healing occurs in animals treated with microparticle loaded hydrogels containing growth factors that were considerably smaller than that of control groups. Moreover, the histological analysis revealed the absence of reactive or granulomatous inflammatory reaction in skin lesions. The results obtained both *in vitro* and *in vivo* disclosed that these systems and its degradation by-products are biocompatible, contributed to the re-establishment of skin architecture and can be used in a near future for the controlled delivery of other bioactive agents used in regenerative medicine.

Keywords: Wound healing, Drug delivery systems, Biocompatibility, Growth factors, *In vivo* and *in vitro* studies.

4.2. Introduction

Wound healing is an extremely dynamic and interactive biological process [1]. It involves complex interactions of extracellular matrix (ECM) molecules, soluble mediators, various resident cells (fibroblasts and keratinocytes) and infiltrating leukocyte subtypes which, together, act to reestablish the integrity of the damaged tissue and replace the lost one [1]. Usually, three processes are involved in wound healing: (i) contraction of the wound edges; (ii) formation of epithelialized scar and (iii) tissue regeneration [2]. This process is slow and rarely accompanied by a complete structural and functional restoration of skin functions, which has repercussions in the quality of life of millions of people around the world [3]. Skin generally needs to be covered by a wound dressing immediately after it's damaged in order to improve the odds of survival and to minimize the loss of its functions. The application of skin substitutes is aimed for bleeding inhibition, fluid and protein loss prevention, electrolyte disturbance control as well as improving esthetic appearance of the wound site [4]. An ideal wound dressing must be biocompatible and biodegradable, prevent dehydration and have good mechanical properties to allow cell growth. Besides that, it should also be porous to allow diffusion of wastes and nutrients [5]. The modern dressings are mainly classified, according to the materials used in their production, to hydrocolloids, alginates and hydrogels [1].

Hydrogels are three-dimensional (3D) polymeric networks capable of absorbing high amounts of water and/or biological fluids. Such is fundamental for the absorption of the excess of wound exudates. Moreover, hydrogels protect the wound site from a secondary infection, are malleable and promote the healing process, by providing a moisturized wound healing environment [6]. These materials are non-adherent and contribute for surface cooling of the wound, which may lead to a marked reduction in patient pain and therefore have high host acceptability [1]. These systems have also been applied for the controlled drug delivery of therapeutic agents (antimicrobials or growth factors (GFs)) into the affected area [7].

However, despite their attractive physical properties, the amount of drug loaded into hydrogels is limited and the high water content of most of these 3D polymeric matrices often results in relatively rapid release profiles, which limits their application as drug delivery systems. Furthermore, there is also the risk of harmful side-effects for patient due to the exposure to high drug concentrations [7]. Dextran is a natural glucose-containing polysaccharide that is a very versatile starting polymer for hydrogel synthesis [8]. The oxidation of dextran by sodium periodate is an easy and well-known method to functionalize dextran with aldehyde moieties. These aldehyde moieties in conjugation with N-nucleophiles have been tested for the synthesis of pro-drugs, as spacers in enzyme immobilization or for GFs' controlled release [8]. Dextran hydrogels have also been used for the stabilization and delivery of fibroblast GFs for tissue regulation [9]. The limitations of hydrogels used in drug delivery can be overcome by the incorporation of different nano- and micro-devices within their polymeric matrix [10]. These systems protect all unstable biological active compounds from degradation, when in contact with the body fluids, and allow a sustained and targeted release of these molecules. This is

fundamental to decrease the number of therapeutic doses administrated and also increase the therapy effectiveness [11]. Different natural or synthetic polymers, lipids, surfactants and dendrimers have been used for drug carrier production [10]. Among them, polysaccharides such as chitosan and alginate have attracted huge attention from various researchers due to their outstanding physical and biological properties [12]. Chitosan is a deacetylated derivative of chitin, a natural polysaccharide found primarily in exoskeletons of arthropods and some fungi [13, 14]. Chitosan presents characteristics like biocompatibility, biodegradability and pH sensitivity that are fundamental for its application as a drug carrier [11, 13]. In recent studies, chitosan has been used to deliver bioactive molecules such as GFs [15].

Different GFs like epidermal growth factor (EGF), basic fibroblast GF, granulocyte–macrophage colony-stimulating factor, human growth hormone–insulin-like GF, platelet derived GF, transforming GF ß and vascular endothelial growth factor (VEGF) have been described in the literature as being involved in the wound healing process [16]. EGF is a single polypeptide comprised of 53 amino acid residues and it has been described that this GF increases the epithelial cell proliferation and the ECM synthesis, which are fundamental to accelerate the wound healing process [17]. VEGF is a multifunctional molecule with important biological activities that depend on both the stage of development and physiological function of the organ, in which it is expressed. It has potent effects on the vascular system, including the ability to stimulate new vessel growth and to increase vascular permeability [18]. Karakeçili et al. reported it as a good candidate to be used in wound healing, due to its specific role in the angiogenesis cascade and its relationship with other GFs and cells [19].

In this study a dextran hydrogel loaded with chitosan microparticles containing VEGF and EGF was produced in order to be used in a near future as a wound dressing.

4.3. Materials and methods

4.3.1. Materials

Adipic acid dihydrazide (AAD), amphotericin B, chitosan (medium molecular weight), dialysis membranes (MWCO ≈ 12,000 Da), Dulbecco's modified Eagle's medium (DMEM-F12), diethylene glycol, EGF, ethylenediaminetetraacetic acid (EDTA), lactate dehydrogenase (LDH) assay, l-glutamine, 3-(4,5-dimethylthiazol-2-yl)-5-(3carboxymethoxyphenyl)-2-(4-sulphofenyl)-2H-tetrazolium, inner salt (MTS), penicillin G, phosphate-buffered saline solution (PBS), sodium periodate, sodium tripolyphosphate (TPP), streptomycin, trypsin and VEGF were purchased from Sigma-Aldrich (Sintra, Portugal). Dextran T500 was purchased from Pharmacia LKB, Sweden. Human fibroblast cells (Normal Human Dermal Fibroblasts adult, cryopreserved cells) were purchased from PromoCell (Labclinics, S.A.; Barcelona, Spain). Fetal bovine serum (FBS) was purchased from Biochrom AG (Berlin, Germany).

4.3.2. Methods

4.3.2.1. Chitosan microparticles preparation

Microparticles were prepared by ionotropic gelation between the positively charged chitosan and the negatively charged TPP ions, as previously reported in the literature [20]. An aqueous solution of chitosan 1.5% (w/v) was prepared by dissolving chitosan in a 1% acetic acid solution [21]. Then, different amounts of EGF, VEGF and EGF + VEGF were dissolved in various chitosan solutions and mixed for 1 h. Microparticle production was performed by using an electrospinning apparatus. The previously prepared solutions were loaded separately into a 10 mL plastic syringe with a needle of 23 gauge at room temperature. The needle was connected to a high-voltage generator (CZE 1000R, Spellman, UK) at a voltage of 9 kV and an aluminum foil was used as the counter electrode. The solution feed rate was controlled through a syringe pump (KD Scientific, KDS-100, Sigma) at a flow of 10 mL/h [21]. Subsequently, the microparticles were collected and washed with distilled water.

4.3.2.2. Dextran hydrogel synthesis

An aqueous solution of dextran (1 g; 0.125% w/v) was oxidized with 2 mL of sodium periodate solution (165 mg/mL) at room temperature, in accordance with a procedure previously described in the literature [8]. The reaction was stopped after 4 h, by adding 10% (v/v) of diethylene glycol. The solution was then dialyzed for 3 days against Milli-Q water, using a dialysis membrane and then lyophilized for 72 h (ScanVac CoolSafe Freeze Drying, LaboGene™, Denmark).

The oxidized dextran (DeOx) at 10% (w/w) was solubilized in PBS. Then, to prepare the hydrogel, 250 µL of DeOx solution was mixed with 250 µL of AAD solution at 15% (w/w) for 30 min [8, 22]. Microparticles loaded with/without GFs were added to DeOx samples before their complete reticulation with AAD. The final concentration of each GF was 10 µg/mL in all tested sample [23].

4.3.2.3. Proliferation of human fibroblast cells in the presence of the carriers

Human fibroblast cells were seeded in T-flasks of 25 cm^2 with 6 mL of DMEM-F12 supplemented with heat-inactivated FBS (10% v/v) and 1% antibiotic/antimycotic solution. After the cells attained confluence, they were subcultivated by a 3–5 min incubation in 0.18% trypsin (1:250) and 5 mM EDTA. Then, the cells were centrifuged, resuspended in culture medium and then seeded in T-flasks of 75 cm^2. Hereafter, the cells were kept in culture at 37 C, in a 5% CO_2 humidified atmosphere inside an incubator [8, 14]. To evaluate cell behavior in the presence of the carriers, each formulation of hydrogel with microparticles was added (n = 5) into a 96-well cell culture plates, in amounts that never exceeded 50 µL of hydrogel and 10 µg/mL of different GFs per well. The materials were sterilized by UV exposure for at least 30 min. Then, DMEM-F12 was added to each well and was left in contact with the carriers for 24 h. Meanwhile,

human fibroblast cells were cultured in 96-well plates at a density of 5 ×10^4 cells per well. After 24 h, the cell culture medium was removed and replaced by the one that was in contact with polymers. This procedure was repeated for 3 days. Cell growth was monitored using an Olympus CX41 inverted light microscope (Tokyo, Japan) equipped with an Olympus SP-500 UZ digital camera.

4.3.2.4. **Characterization of the cytotoxic profile of the carriers**

To evaluate the cytotoxicity of the carriers, human fibroblast cells were seeded at a density of 5 × 10^4 cells per well, and cultured with DMEM-F12. At the same time, in another plate, the culture medium was added to the sterilized polymers, and left there for 24 and 48 h. After, the cell culture medium was removed and replaced with 100 µL of medium that was in contact with the carriers. Then the cells were incubated at 37 C in a 5% CO_2 humidified atmosphere for another 24 h. Subsequently, cell viability was assessed through the reduction of the MTS into a water-soluble brown formazan product (n = 5), by an adaptation of the method previously described in the literature [8]. The medium of each well was then removed and replaced with a mixture of 100 µL of fresh culture medium and 20 µL of MTS/PMS reagent solution. The cells were incubated for 4 h at 37 C, under a 5% CO_2 humidified atmosphere. The absorbance of the produced formazan was measured at 492 nm using a microplate reader (Sanofi Diagnostics Pasteur). Wells containing cells in the culture medium without materials were used as negative control (live cells). Ethanol 96% was added to wells containing cells as a positive control (dead cells) [14, 24, 25]. Furthermore, a LDH assay was also performed to evaluate the amount of extracellular LDH released from damaged cells to the extracellular medium [26]. After 24 and 48 h in the presence of carriers, the well plates were shaken briefly and 50 µL of culture medium was transferred into a fresh 96-well plate. Then, the LDH assay mixture (100 µL) was added to each well. After 20–30 min, the enzymatic activity was stopped by the addition of chloride acid (HCl). Then, the absorbance of the samples was measured at 492 nm [27]. Wells containing cells in the culture medium without carriers were used as negative control (live cells) [28].

4.3.2.5. **Scanning electron microscopy analysis**

The morphology of the microparticles and hydrogel with/without adhered human fibroblast cells was characterized by scanning electron microscopy (SEM). Cells (6 × 10^4 cells/well) were seeded with sterilized chitosan microparticles and DeOx with/without microparticles in 48-well plates, over a coverslip, for 48 h. Samples were fixed with 2.5% glutaraldehyde overnight and then frozen in a glass container using liquid nitrogen and freeze-dried for 3 h. Finally, the carriers were mounted on an aluminum board using a double-sided adhesive tape and covered with gold using an Emitech K550 (London, England) sputter coater. The samples were then analyzed using a Hitachi S-2700 (Tokyo, Japan) scanning electron microscope operated at an accelerating voltage of 20 kV and at various amplifications[14, 29].

4.3.2.6. *In Vivo* Assays

A total of 30 *Wistar* rats (8–10 weeks) weighing between 150 and 200 g were used in wound healing studies. The animal protocols followed in the present study were performed according to the guidelines set forth in the National Institutes of Health Guide for the care and use of laboratory animals. The animals were individually anesthetized with an intra-peritoneal injection of ketamine (40 mg/kg) and xylazine (5 mg/kg) for surgery and induction of the burn wound. The operative area of skin was shaved and disinfected using ethanol (96%) and the dorsal skin of the animals was exposed to water at 95 ± 1 C, for 10 s. Wounds of 2 cm diameter were created with no visible bleeding [14]. The animals were divided into six groups: in group 1 wounds were filled with EGF + VEGF dissolved in PBS; in group 2 wounds were filled with DeOx loaded with chitosan microparticles (DeOx + Ch) without GFs; in group 3 wounds were filled with DeOx loaded with microparticles with VEGF incorporated (DeOx + VEGF); in group 4 wounds were filled with DeOx with microparticles loaded with EGF (DeOx + EGF); group 5 was used as control and wounds were covered with PBS; the wounds in group 6 were filled with DeOx loaded with microparticles containing EGF + VEGF (DeOx + (EGF + VEGF)). Group 1 was treated every two days while the others were treated every 7 days. Then, the animals were kept in separate cages and were fed with commercial rat food and water *ad libitum*. All the animals showed good general health condition throughout the study, as assessed by their weight gain. The animals were sacrificed after 7, 14 and 21 days [14].

4.3.2.7. Histological study

Tissue specimens were obtained from the wound area by sharp dissection at days 7, 14 and 21. The samples from skin lesions and organs (brain, heart, lung, liver, spleen and kidney) were obtained by necropsy and were formalin fixed and paraffin embedded for routine histological processing. A 3 μm section obtained from each paraffin block was stained with hematoxylin and eosin (H&E) and evaluated using a light microscope with specific image analysis software from Zeiss. Skin fragments with no carriers and GFs were used as control. The assessment of the brain, lung, liver, spleen and kidney samples was performed in order to check for any morphological alteration[14].

4.3.2.8. Evaluation of the wound size

Images of the wound area were taken with a digital camera (Nikon D50) and analyzed with image analysis software ImageJ (Scion Corp., Frederick, MD). Measurement of the wound closure area was defined by the limits of grossly evident epithelialization, with all surface areas in a two-dimensional plane calibrated against the adjacent metric ruler. The percentage of wound size (WS) was calculated using the following formula (1):

$$WS = D_N/D_0 \times 100\ (\%)\ (1)$$

where D_0 is the dimension of the full thickness circular skin wound area (2 cm diameter) on day 0, and D_N is the dimension of the wound area on the indicated day[14].

4.3.2.9. Statistical analysis

Statistical analysis was performed using one-way ANOVA with Dunnett's post hoc test. Computations were performed using a MYSTAT 12 statistical package (Systat Software, a subsidiary of Cranes Software International Ltd.).

4.4. Results

4.4.1. Characterization of the morphology of the carriers

Freeze-dried DeOx showed a spongy morphology similar to that of cotton, as can be observed in figure 4.1a. Subsequently the hydrogel was prepared in Teflon molds and impregnated with microparticles (figure 4.1b).

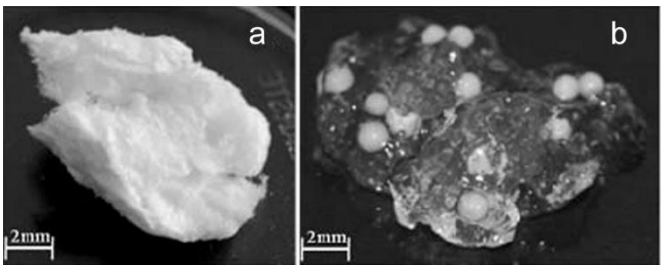

Figure 4.1: Images of freeze-drying oxidized dextran (a) and dextran hydrogel with microparticles incorporated (b).

SEM analysis of DeOx hydrogel (figure 4.2a) revealed a highly porous and interconnected surface. Chitosan microparticles produced by electrospraying presented a spherical shape and an average diameter of approximately 255 ± 0.9 µm (figure 4.2b). Figure 4.2c shows that the chitosan carriers produced had a slightly smoother surface, in accordance with what was previously reported in the literature [30, 31].

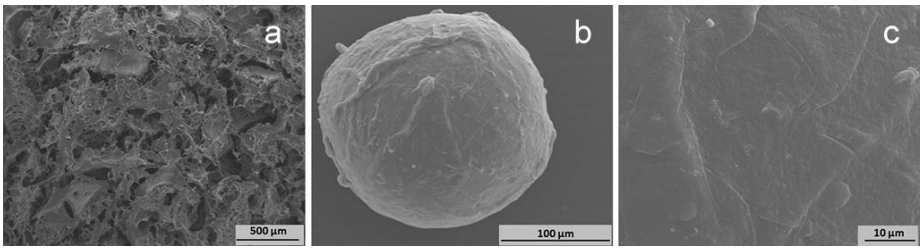

Figure 4.2: SEM images of surface of oxidized dextran 50x (a), chitosan microparticles 400x (b) and chitosan microparticles 2000x (c).

4.4.2. Evaluation of the cytotoxic profile of the carriers

To assess the applicability of our hydrogel for the envisioned biomedical application, the cytocompatibility of dextran hydrogel loaded with chitosan microparticles with/without GFs incorporated was first characterized through *in vitro* studies. Cell adhesion and proliferation were observed in wells where cells were in contact with different carriers (figure 4.3) and in the negative control (cells without biomaterials), at the predetermined time points. Dead cells with their typical spherical shape were visualized in the positive control (ethanol treated cells). The observation of cell adhesion and proliferation in the presence of the carriers showed that all of them are biocompatible.

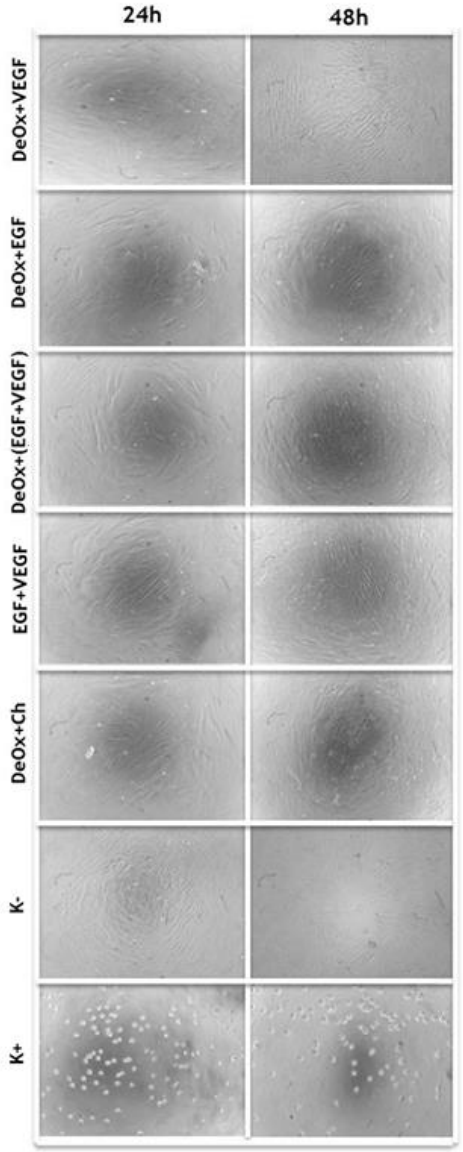

Figure 4.3: Microscopic photographs of human fibroblast cells after being seeded in the presence of the carriers during 24 h and 48 h. DeOx + VEGF: oxidized dextran loaded with chitosan microparticles with VEGF incorporated; DeOx + EGF: oxidized dextran loaded with chitosan microparticles with EGF incorporated; DeOx + (EGF + VEGF): oxidized dextran loaded with chitosan microparticles with VEGF and EGF incorporated; VEGF + EGF: VEGF and EGF dissolved in cultured medium; DeOx + Ch: oxidized dextran loaded with chitosan microparticles; negative control (live cells); positive control (death cells). Original magnification 100×.

SEM images were also acquired to further examine and characterize cell adhesion to the materials. Cell growth and filopodia were observed, indicating that cells adhered and grew on hydrogels surface after 48 h (figure. 4.4).

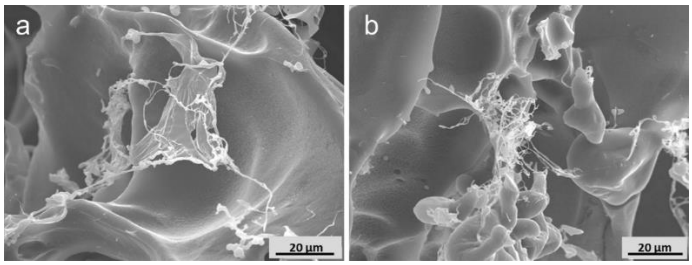

Figure 4.4: SEM images of human fibroblast cells in contact with oxidized dextran hydrogel. Original magnification 1000x (a) and oxidized dextran hydrogel with chitosan microparticles incorporated. Original magnification 1000x (b).

To further assess the biocompatibility of the carriers, MTS and LDH assays were also performed. Both of these assays showed that cells remained viable in contact with all tested samples (with and without the GFs) after 24 and 48 h of incubation (figure 5). These results clearly demonstrate that these vehicles are biocompatible and may be used for GF delivery systems for wound healing.

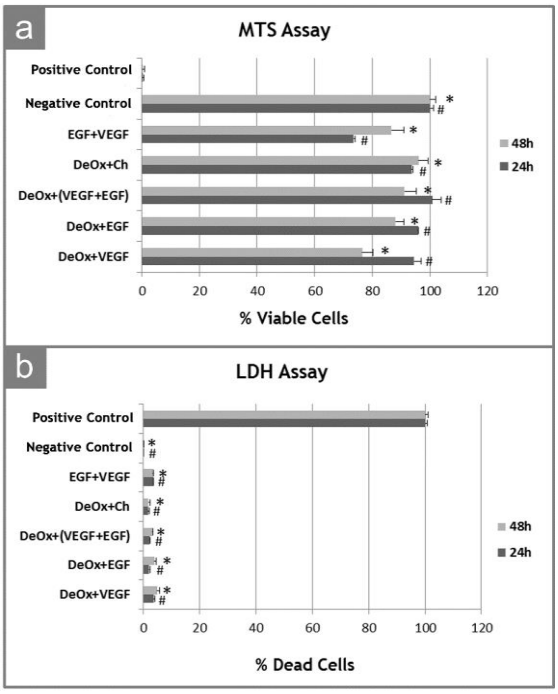

Figure 4.5: Cellular activities measured by the MTS assay (a) and cellular integrity measured by the LDH assay (b) after 24 h and 48 h. Positive control (death cells); negative control (live cells); VEGF and EGF (VEGF + EGF); oxidized dextran loaded with chitosan microparticles (DeOx + Ch); oxidized dextran loaded with chitosan microparticles with VEGF and EGF incorporated (DeOx + (EGF + VEGF)); oxidized dextran loaded with chitosan microparticles with EGF incorporated (DeOx + EGF); oxidized dextran loaded with chitosan microparticles with VEGF incorporated (DeOx + VEGF). Each result is the mean ± standard error of the mean of at least three independent experiments. Statistical analysis was performed using one-way ANOVA with Dunnett's post hoc test ($^{*}p < 0.05$; $^{\#}p < 0.05$).

4.4.3. *In vivo* evaluation of the wound healing process

For the evaluation of the in vivo wound healing process Wistar rats were used and divided into six groups, as previously described in Section 4.3.2.6. Groups 1–5 were set as controls. Group 1 was used to check if skin regeneration was correlated with the direct application of multiple GFs (EGF + VEGF). The application of dextran hydrogel loaded with chitosan microparticles without GFs (group 2) was used to study whether the effect on wound healing was due to the use of GFs or to the biomaterials used for carrier production. Groups 3 and 4 were used to compare the application of GFs alone or in a combined mode. Group 5, where the wounds were only treated with PBS, was used to determine whether wound contraction occurred due to the combined use of biomaterials/GFs. Finally, group 6 was set as a test group where a synergistic combination of DeOx hydrogel and chitosan microparticles loaded with EGF + VEGF was used to study the influence of the system loaded with two GFs in the wound healing process. In vivo experiments showed that hydrogel carriers promoted moist healing, as previously reported in the literature[1]. Figure. 4.6 shows a set of typical wound beds after the surgical procedure and application of the hydrogels. The healing patterns were observed after 2, 5, 9, 12, 16 and 21 days.

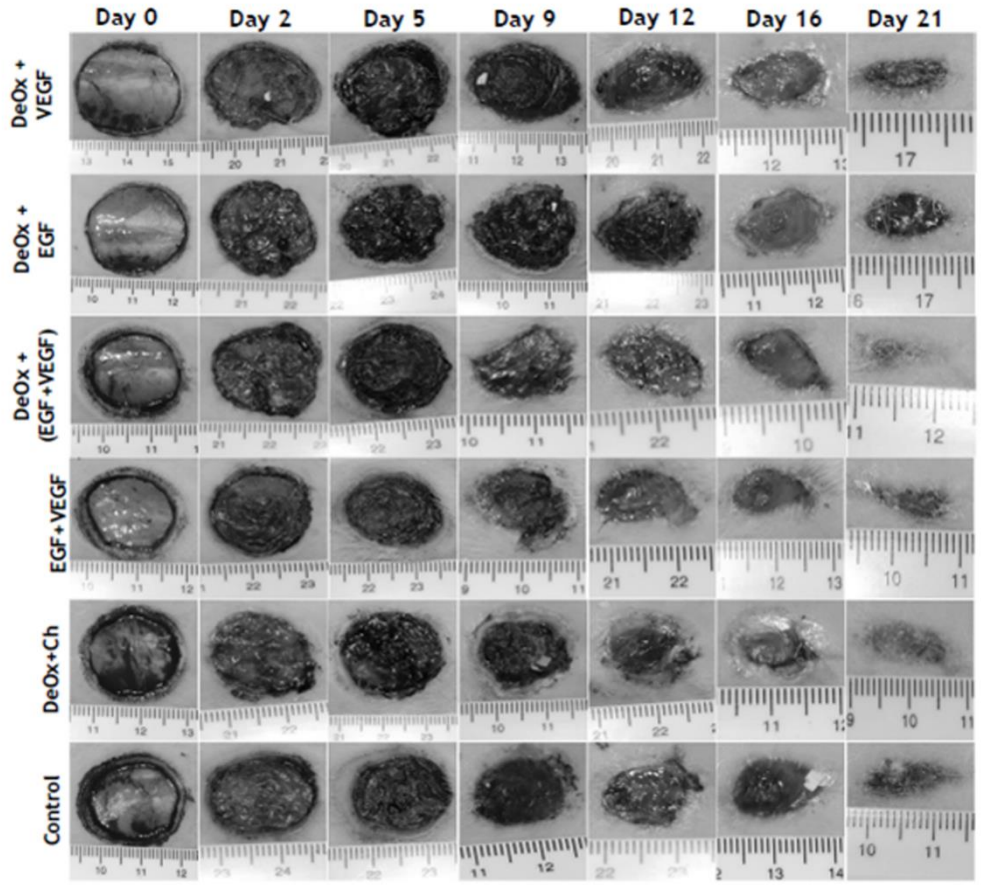

Figure 4.6: Macroscopic images of the wound-healing panorama over 21 days. A deep third-degree burn wound with 2 cm diameter was induced at the dorsal skin of each female Wistar rat. Digital images were acquired after the 2nd, 6th, 9th, 12th, 16th and 21st days of the injury.

In figure. 4.7, the evolution of the wound size for the different groups over time is presented. From the analysis of this figure, it can be inferred that the best results were obtained for the group treated with DeOx loaded with microparticles containing EGF + VEGF, since the wound closure occurred before.

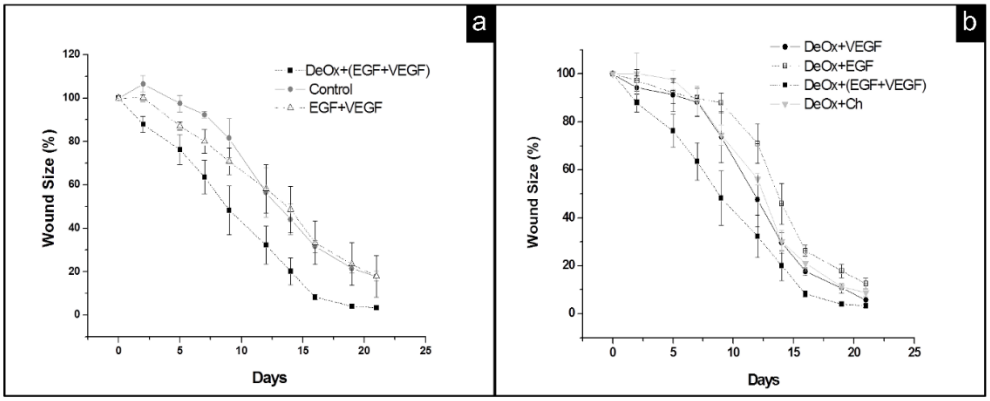

Figure 4.7: Effect of DeOx + (EGF + VEGF) on burn wound compared to the control group and EGF + VEGF (a), DeOx + (EGF + VEGF) on burn wound compared to the DeOx + VEGF, DeOx + EGF and DeOx + Ch (b). The surface area of the burn wounds was calculated as described in Methods and reported at each time point as the percentage of the surface area at baseline. Each point represents the mean ± standard error of the mean of at least three independent experiments.

4.4.4. Histological analysis

Figure. 4.8 presents the histological data obtained in this study. From its observation, it can be concluded that the granulation tissue layer and epithelial layer thickness increased progressively from days 7 to 21. No specific inflammation or reactive granulomas were observed due to the presence of DeOx, chitosan and GFs in all groups. No pathological abnormalities were observed in the brain, lung, liver, spleen or kidney samples (data not shown).

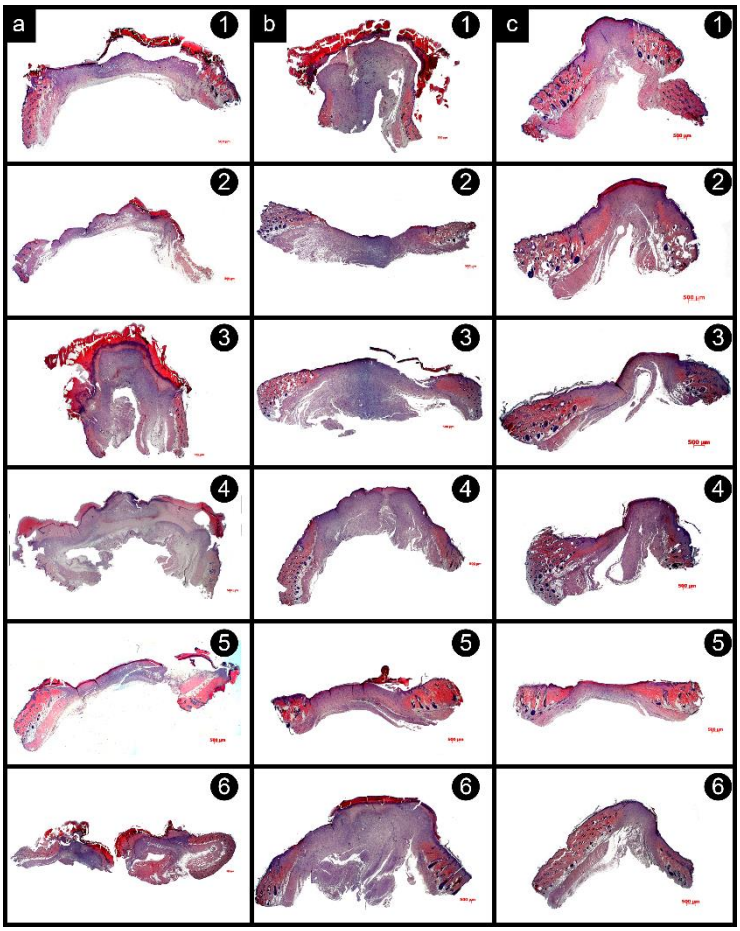

Figure 4.8: Hematoxylin and eosin-stained sections of biopsies for the morphological evaluation of skin lesions after 7 days, scale bar 500 μm (a), 14 days, scale bar 500 μm (b) and 21 days, scale bar 500 μm (c). Group 1 treated with EGF + VEGF (1), group 2 treated with DeOx + Ch (2), group 3 treated with DeOx + VEGF (3), group 4 treated with DeOx + EGF (4), group 5 treated with PBS (5) and group 6 treated with DeOx + (EGF + VEGF) (6).

4.5. Discussion

Skin engineering methodologies require biomaterials that promote the reconstruction of the architecture of native skin, which is sometimes irreversibly destroyed by injuries or diseases [32]. In our study, a DeOx based-hydrogel was produced to be used in a near future as a skin substitute.

Dextran is biocompatible and can be degraded through the action of dextranases in various organs of the human body, including liver, spleen, kidney and colon [8]. Keeping in mind the wound dressing application, the porous section of DeOx (figure. 4.2a) promotes drainage of the wound, prevents the build-up of exudates, and may be an optimum wound bed for autografting. It can also increase the surface area-to-volume ratio of hydrogel scaffolds promoting cell growth, tissue invasion, local angiogenesis and facilitate nutrient transport, which is fundamental for the wound-healing process [14]. These results are in agreement with experimental data obtained by other researchers [33, 34].

In order to increase the period among which GFs were released from the DeOx based hydrogels, chitosan microparticles loaded with these bioactive molecules were incorporated into this polymeric matrix, since first GFs have to be released, in a controlled form, by chitosan microparticles to the hydrogel and then from the hydrogel to the surrounding environment. Such strategy allowed to increase the period among which the GFs are released as previously described for other polymers and other particle-based delivery systems [35, 36].

GFs (EGF and VEGF) were chosen since they are actively involved in the natural skin regeneration process [16]. In the present study, their overall contribution for the healing process was evaluated when they were used alone or both at the same time, within drug delivery systems

Chitosan microparticles were produced by an electrospraying method (figure 4.2b), which is a slightly modified form of the electrospinning process. It allows the production of particles with smaller diameters, from micrometers to nanometers [35]. The chitosan microparticles produced presented a spherical shape, an average diameter of approximately 255 ± 0.9 μm, and a slightly smoother surface when compared with microparticles produced by traditional methods [37]. The combination of these two systems (hydrogel and microparticles) was advantageous since it allows wound protection against toxins and microorganisms and also avoids dehydratation of the patient. Furthermore, the hydrogel acted as a support for the carriers incorporated in its polymeric matrix and increased the period over which GFs were released. The DeOx hydrogel produced showed a highly porous internal structure, with a pore size sufficiently large to accommodate fibroblasts, which is crucial for skin regeneration, as previously described by Weng and collaborators [34].

The results of the *in vitro* studies showed that cell adhered and proliferated after 48 h of being seeded in the presence of the carriers. These results were corroborated with that obtained in the MTS and LDH assays. Furthermore, the LDH results demonstrated that human fibroblast membrane integrity was not affected when in contact with carriers. These results were expected since the different components of the system developed have been previously tested individually in other studies [8,14].

Subsequently, these carriers were further characterized through *in vivo* studies. The wound area of animals treated with PBS (group 5) increased during the first days, while for other groups it did not. Such result emphasizes the importance of an initial covering of the damaged area, as already described in literature [38].

The healing process was slower for animals from group 1, which received several doses of GFs every two days, than for those of group 6 (treated with a single dose per week). These results show an asset for the use of this system since it can, simultaneously, reduce costs and pain associated with skin regeneration. Furthermore, the granulation tissue layer and the epithelial layer thickness increased faster for these two groups, which can be explained by the formation of new blood vessels in dermis layer of these animals (figure 4.8). These findings are in accordance with previous studies, describing that this set of biomaterials may aid in the re-establishment of native tissue architecture [34].

In group 2, in which animals were treated with DeOx loaded with microparticles without GFs, the wound healing was slower than for groups 1 and 6. Such result was expected, since GFs play key roles in the regulation on skin regeneration [19]. On the other hand, hydrogel avoids tissue dehydration and bacterial contamination and also circumvents exuberant inflammatory response.

Groups 3 (DeOx + VEGF) and 4 (DeOx + EGF) presented similar results. The healing process for these groups occurred at a slower rate than that observed for group 6. Such demonstrates that the combined use of GFs improves the establishment of the regenerative cascade in order to produce new extracellular matrix and promote angiogenesis that are fundamental for skin regeneration [39].

In studies of skin regeneration, the analogy between experimental model and human skin is important and relevant [40]. Like for the case of human burns, the thermal injury in rats skin destroys epidermis, dermis and hypodermis [41]. In our study, the lack of a reactive or a granulomatous inflammatory reaction in skin lesions treated with biomaterials and the absence of pathological abnormalities in the organs obtained by necropsy supported the local and systemic histocompatibility of the biomaterials.

In this work, a versatile, non-toxic, *in situ* crosslinkable biodegradable dextran hydrogel was produced to be used as a wound dressing in the first phase of skin regeneration. The results obtained both in the *in vitro* and *in vivo* assays demonstrated the biocompatibility of the synthesized vehicles, thus, suggesting that AAD can be used as a crosslinking agent for DeOx hydrogel production, as previously reported by Maia et al. [8]. The *in vivo* studies demonstrated that the application of this system improves the mechanical, chemical and biological protection of the damaged skin. Moreover, the incorporation and spatiotemporally controlled release of VEGF and EGF also improve angiogenesis (VEGF), and re-epithelialization (EGF) that are crucial for the reestablishment of native tissue architecture [42]. Further studies are currently being undertaken to evaluate the applicability of these systems as skin substitutes in diabetic rats.

4.6. Conclusion

A versatile, non-toxic, *in situ* crosslinkable, biodegradable hydrogel has been successfully prepared with DeOx in order to be used as a wound dressing. The *in vitro* assays revealed that hydrogel loaded with microparticles both with and without the GFs is non-cytotoxic. The *in vivo* assays suggested that dextran hydrogel and chitosan microparticles with the two GFs encapsulated promote faster wound healing with no signs of local or systemic inflammatory response. The results obtained here support the simultaneous application of the two GFs, with synergic roles in wound healing mechanism. Moreover, chitosan microparticles were considered good vehicles to deliver the GFs studied, since a unique application per week of DeOx loaded with GFs helps to reduce the wound area faster than when free EGF + VEGF was applied every

two days. Furthermore, dextran hydrogel could be adapted to be used as an *in situ* gelable wound dressing.

In the near future these two systems (hydrogel and microparticles) will also be used as carriers for other GFs or for cell encapsulation, widening the applicability of these devices to other areas of regenerative medicine.

4.7 Acknowledgments

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Chapter 5
Paper III

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Carbohydrate Polymers

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Thermoresponsive chitosan-agarose hydrogel for skin regeneration

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5.1. Abstract

Healing enhancement and pain control are critical issues on wound management. So far, different wound dressings have been developed. Among them, hydrogels are the most applied. Herein, a thermoresponsive hydrogel was produced using chitosan (deacetylation degree 95%) and agarose. Hydrogel bactericidal activity, biocompatibility, morphology, porosity and wettability were characterized by Confocal Microscopy, MTS assay and SEM. The performance of the hydrogel in the wound healing process was evaluated through *in vivo* assays, during 21 days.

The attained results revealed that hydrogel has a pore size (90-400 μm) compatible with cellular internalization and proliferation. A bactericidal activity was observed for hydrogels containing more than 188 μg/mL of chitosan. The improved healing and the lack of a reactive or a granulomatous inflammatory reaction in skin lesions treated with hydrogel demonstrate its suitability to be used in a near future as a wound dressing.

Keywords: Antimicrobial activity, Biocompatibility, Skin substitutes, Wound healing.

5.2. Introduction

Burned patients may experience a wide number of potentially fatal complications including shock, infection, electrolyte imbalances and respiratory failure [1]. Furthermore, they can also experience severe psychological and emotional distress due to long periods of hospitalization, scarring and deformity [2]. Such, highlights the importance of developing new wound dressings that improve the healing process, making it less painful and, simultaneously, contribute for the reestablishment of skin structure and functions in a shorter period of time [3, 4].

Among the different wound dressers produced so far, hydrogels due to their intrinsic properties are the ones that better mimic the extracellular matrix (ECM) and have the potential to direct cell migration, adhesion and growth during tissue regeneration, events that are crucial for skin regeneration [5-7]. When applied at the wound site hydrogels promote a moist healing and cool the surface of the wound, which may lead to a relevant reduction in pain and therefore have high patient acceptability [8, 9]. Some hydrogels have the particularity of gelling within the desired tissue or body cavity as a result of polymer interactions. Such *in situ*-forming systems advantageously flow freely as injectable liquids before administration and gel under physiological conditions. Temperature-sensitive systems that gel at body temperature are especially attractive [10].

Herein, the main goal of this study was to produce a new *in situ* thermoresponsive hydrogel composed by agarose and chitosan to be used as an injectable scaffold for tissue regeneration. As described above *in situ* formed hydrogels are mouldable, i.e., are able to acquire the right shape at the wound site, without wrinkling or fluting and interacting with the damaged tissue. Agarose is a biocompatible linear polysaccharide extracted from marine algae [11], consisting of 1, 4-linked 3,6-anhydro-α-L-galactose and 1,3-linked ß-D-galactose derivatives that forms thermorreversible gels with suitable properties for tissue engineering applications [12]. The mechanical properties presented by agarose are similar to those of tissues and can be easily tailored by varying polymer concentration. When solubilized in water, it forms a gel with a rigid network, resulting on a three-dimensional porous structure providing a good environment for cell adhesion, spreading and proliferation [13-16].

Furthermore, agarose hydrogels may be polymerized *in situ* reducing invasiveness of the surgery and also allow the hydrogel to acquire the required shape [17].

Chitosan, the partially acetylated cationic (1-4)-2-amino-2-deoxy-ß-D-glucan, is industrially produced from marine chitin. Its regenerative properties have been amply recognized [18]. In acidic aqueous solutions the protonated free amino groups of glucosamine promote the solubility of this polymer. Its hydrophilic surface promotes cell adhesion, proliferation and differentiation [19-21]. Chitosan derivatization allows an extensive adjustment of mechanical and biological properties, contributing for its anticholesterolemic and antimicrobial activity, biocompatibility, biodegradability, hemostasis and capacity to stimulate the healing process [22-25].

The interaction of chitosan and agarose allows the production of hydrogels capable of gelling within the desired site, as a result of polymer interactions.
In this study, deacetylated chitosan was combined with agarose in order to explore the polymeric interactions and the thermosensitive character of agarose for producing a hydrogel. The produced hydrogel was characterized through *in vitro* and *in vivo* assays, in order to evaluate its suitability for being used as a wound dressing.

5.3. Materials and Methods

5.3.1. Materials

Agarose (low melting point-ultrapure grade) was acquired from Nzytech (Lisboa, Portugal). Amphotericin B, Bovine serum albumin (BSA), Chitosan (medium molecular weight (MMW) (degree of deacetylation: 83.35% ±0.23); Dulbecco's modified Eagle's medium (DMEM-F12), Ethylenediaminetetraacetic acid (EDTA), LB Broth, Kanamycin, N-acetyl-D-glucosamine, Phosphate-Buffered Saline Solution (PBS), Resazurin (7-hydroxy-3H-phenoxazin-3-one-10-oxide), Streptomycin and Trypsin were purchased from Sigma-Aldrich (Sintra, Portugal). Acetic acid and Sodium hydroxide were bought to Pronalab (Barcelona, Spain). Normal Human Dermal Fibroblasts adult (NHDF), criopreserved cells were purchased from PromoCell (Labclinics, S.A.; Barcelona, Spain). *Staphylococcus aureus* (*S. aureus*) ATCC 25923 was used to evaluate antimicrobial properties of hydrogel. Fetal bovine serum (FBS) (free from any antibiotic and heat inactivated) was acquired from Biochrom AG (Berlin, Germany). 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was purchased from Promega (Canada, USA). Tris Base was obtained from Fisher Scientific (Portugal).

5.3.2. Synthesis and Characterization of deacetylated Chitosan

Chitosan was deacetylated by adapting a method previously described in literature [26] and then purified. The recovered chitosan was dissolved in 1 M acetic acid solution filtered with a 0.22 µm filter to remove traces of any solid particles. Afterwards, the pH was adjusted to 7 with 1M NaOH, which resulted in the precipitation of the chitosan material. The product was then centrifuged at 4500 rpm (Sigma 3K18C centrifuge), this procedure was repeated three times and finally the recovered pellet was lyophilized for one day. The degree of deacetylation was measured by using the first derivative UV-spectroscopy (1DUVS) method [27]. UV–vis chitosan spectra were obtained using a Shimadzu 1700 UV–vis spectrophotometer. Table S1 shows the degree of deacetylation for the commercial and deacetylated chitosan samples. The chitosan used for hydrogel production had a deacetylation degree of 95.08% ±0.48, meaning that almost all of the primary amine groups of the chitosan polymer chain are positively charged.

5.3.3. Production of Chitosan-Agarose hydrogel

For Chitosan-Agarose hydrogel (CAH) production, different percentages of deacetylated chitosan (from 0.75% to 2.5%), previously filtered with a 0.22 μm filter, were initially dissolved in 1 % acetic acid solution. Then, agarose powder was added to the chitosan solution, under stirring at 50ºC, until reaching a final concentration of 3%, w/v (please see Table S2 for further details).

5.3.4. Determination of contact angle of CAH

Contact angles of CAH (3% agarose - 0.75% chitosan and 3% agarose - 1.5% chitosan) were determined using a Data Physics Contact Angle System OCAH 200 apparatus, operating in static mode. For each sample, water drops were placed at various locations of the materials surface, at room temperature (RT). The reported contact angles are the average of at least three measurements.

5.3.5. Study of water uptake ability (swelling)

To evaluate CAH (3% agarose - 1.5 % chitosan) water uptake ability, a known weight (W_0) of the hydrogel was immersed in 1 mL Tris buffer, at pH 5 and 37°C (n=5). At predetermined intervals the swollen CAH was removed from the solution, the excess of water removed with filter paper, and subsequently weighted (W_t) [28]. The swelling ratio was evaluated by using Equation 1:

$$Swelling\ ratio\ (\%) = \frac{Wt - Wo}{Wo} * 100$$

Where W_t is the final weight and W_0 is the initial weight of CAH.

5.3.6. Proliferation of human fibroblast cells in the presence of CAH

To evaluate NHDF growth in the presence of CAH, cells were seeded in 96-well plates, containing the hydrogel, at a density of $2x10^4$ cells/cm^2 per well, and incubated at 37°C in a 5% CO_2 humidified atmosphere, for 24 and 72h. Cell growth was monitored by using an Olympus CX41 inverted light microscope (Tokyo, Japan) equipped with an Olympus SP-500 UZ digital camera.

5.3.7. Characterization of the cytotoxic profile of CAH

To evaluate NHDF cell viability in the presence of CAH an MTS assay was used. First, $2x10^4$ cells per well were seeded on CAH surface. After 24 and 72h of incubation at 37°C, the culture medium was removed and replaced by a mixture of 100μL of fresh culture medium and 20μL of

MTS/PMS (phenazine methosulfate) reagent solution. Then, cells were incubated for 4h, at 37°C, under a 5% CO_2 humidified atmosphere. Subsequently the absorbance was measured at 492nm using a microplate reader (Sanofi, Diagnostics Pauster). Ethanol 96% was added to cells to be used as positive controls (K^+), whereas cells without biomaterials were used as negative controls (K^-) [29-32].

5.3.8. Scanning electron microscopy analysis

CAH (3% agarose - 0.75% chitosan and 3% agarose - 1.5% chitosan) morphology with/without adhered NHDF cells was characterized by scanning electron microscopy (SEM), using a procedure previously described elsewhere [29]. Briefly, samples were dehydrated in graded ethanol (ETOH) of 70, 80, 90, and 100%, 5 minutes each. Then, hydrogels were mounted on an aluminium board using a double-sided adhesive tape and sputter coated with gold using an Emitech K550 (London, England) sputter coater. A Hitachi S-2700 (Tokyo, Japan) scanning electron microscope operated at an accelerating voltage of 20 kV and at various magnifications [29], was used for samples analysis.

5.3.9. Confocal microscopy analysis

For the visualization of NHDF cells within CAH (3% agarose - 1.5% chitosan), $10x10^3$ cells/mL were seeded in µ-Slide 8 well Ibidi imaging plates (Ibidi GmbH, Germany) in contact with hydrogel. After 24h, cells were fixed with 4% paraformaldehyde (PFA) in PBS for 20 min and then stained with 1µL of Propidium Iodide (PI) (1mg/mL) during 15 min, at 37ºC. Imaging experiments were performed in a Zeiss LSM 710 laser scanning confocal microscope (CLSM) (Carl Zeiss SMT Inc., USA), where consecutive z-stacks were acquired. 3D reconstruction and image analysis was performed in Zeiss Zen 2010 [33, 34].

5.3.10. Characterization of the antibacterial properties of CAH

5.3.10.1. Determination of Minimum Inhibitory Concentration of CAH

S. aureus, a Gram-positive bacteria, was used to evaluate the antimicrobial properties of CAH. *S. aureus* ($1x10^6$ colony-forming units (CFU)/mL) was inoculated in culture medium (LB Broth). Then, several formulations of CAH with 3% agarose and different concentrations of Chitosan (125–400 µg/mL) were tested. A negative (without CAH) and a positive control (containing Kanamycin antibiotic (30 mg/mL)) were also prepared. Then, the 96-well plate was incubated for 24h, at 37°C. For monitoring bacterial growth, 10 µL of resazurin (0.1%) was added and, after 24h, the fluorescence was measured using a fluorescence plate reader with filter set Ex545/Em590 [35].

5.3.10.2. Evaluation of biofilm deposition at CAH surface

S. aureus proliferation at CAH surface was also evaluated by SEM analysis [36]. Defined concentrations of CAH were placed on the surface of a plate of LB agar, in contact with *S. aureus* ($1x10^8$ CFU/mL), without any other antimicrobial agent. Then the petri plate was incubated for 24h, at 37°C. After, the morphologies of CAH with/without *S. aureus* were analyzed by acquiring SEM images.

5.3.11. In vivo assays

To perform the *in vivo* assays, a total of 10 Wistar rats (8–10 weeks) weighing between 150 and 200 g were used. The animal protocols followed in the present study were performed according to the guidelines set forth in the National Institutes of Health Guide for the care and use of laboratory animals. The experimental setup was performed according to that previously used by Ribeiro and collaborators [29]. Animals were separated into two groups: in group 1, used as control, wounds were covered with saline solution; whereas in group 2, wounds were filled with CAH (3% agarose- 1.5% chitosan). During the study, animals were kept in separate cages and were fed with commercial rat food and water *ad libitum*.
To follow the wound healing process, animals were photographed with a digital camera (NikonD50) along time. Then the wound size (WS) was determined by using an image analysis software ImageJ (Scion Corp., Frederick, MD). Animals were sacrificed after 7, 14 and 21 days.

5.3.12. Histological analysis

To evaluate the local and systemic immune response of the host to CAH, tissue specimens were obtained from each wound area by sharp dissection at days 7, 14 and 21. The samples of skin tissues were obtained by necropsy, formalin fixed and paraffin embedded for routine histological processing. A 3 μm section was obtained from each sample using a cryostat microtome (Leica CM1900) and then stained with hematoxylin and eosin (H&E). Subsequently, samples were observed using a light microscope with a specific image analysis software from Zeiss. Skin fragments with no hydrogel were used as control. Brain, lung, liver, spleen and kidney samples, obtained by necropsy, were also analysed to check for any morphological alteration [29, 37].

5.4. Results and Discussion

5.4.1. Characterization of the morphology of CAH

The CAH produced in this study gels when the temperature decreases from 50 ºC to 37 ºC, changing from transparent to opaque (see figure 5.1). The thermoresponsive character of CAH is attributed to agarose, that undergoes through a reversible gelling process without losing its

mechanical and thermal properties [38]. In the literature it is described that at high temperatures polymeric chains have a random coil conformation. By lowering temperature they start to form double helices and aggregates that act as physical junctions of the gels [39].

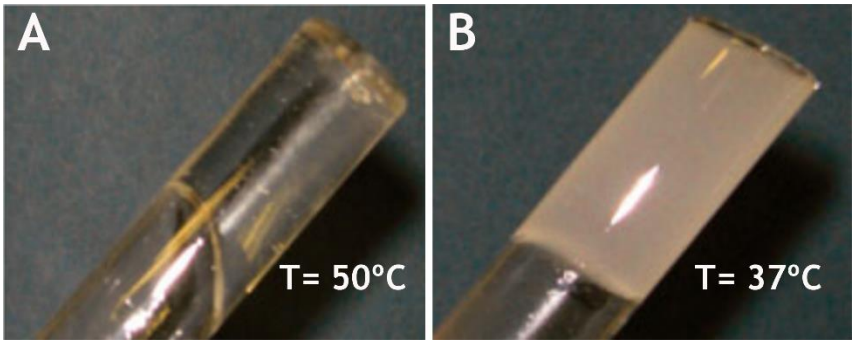

Figure 5.1: Macroscopic images of CAH at 50 °C (A) and 37 °C (B).

When tissue engineering applications are envisioned, cell adhesion to biomaterials is a crucial prerequisite to allow tissue regeneration [40]. Among the different factors affecting cell adhesion, materials surface charge, morphology and porosity are considered the most relevant. SEM images presented in figure 5.2 show that CAH has an irregular surface and a porous interconnected structure, with pore diameters (90-400 μm), that allow a good cell penetration and proliferation in contact with hydrogel, as well as nutrients and oxygen diffusion into the bulk of the matrix [41, 42].

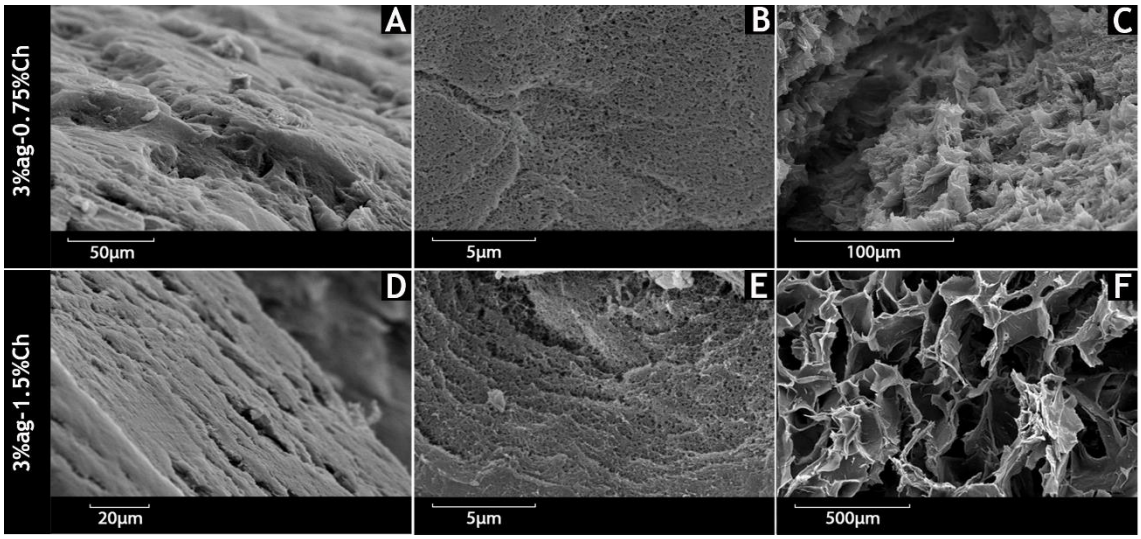

Figure 5.2: SEM images of CAH: cross section of CAH (3% ag–0.75% Ch) (A); surface of CAH (3% ag–0.75%Ch) (B); inner structure of CAH (3% ag–0.75% Ch) (C); cross section of the CAH (3% ag–1.5% Ch) (D); surface of CAH (3% ag–1.5% Ch) (E); inner porous network of CAH (3% ag–1.5% Ch) (F).

5.4.1.1. Contact angle of CAH

To evaluate the hydrophilic character of CAH surface water contact angles were determined. The CAH, 3% agarose – 0.75% Chitosan and 3% agarose – 1.5% Chitosan, presented contact angles of 40.9º± 3.8º and 43.7º± 3.7º, respectively. Such values demonstrate the moderate hydrophilic character of the hydrogels produced. According to what was previously described in literature, the surface is considered hydrophobic when the contact angle is between 90° and 150°and hydrophilic when it is comprehended 10° and 90°. Cell adhesion to materials occurs in optimal conditions when the polymer surfaces present a moderate wettability, with water contact angles ranging 40-70° [43].

5.4.1.2. Study of water uptake ability of CAH

To evaluate the appropriateness of CAH for this biomedical application, its swelling behavior was studied (figure S1). The obtained results demonstrated that the high water uptake capacity of CAH after 12 h, caused an increase in the pore diameter of the polymeric mesh and subsequent diffusion of nutrients, cells, bioactive molecules and waste along hydrogel [44]. Such swelling behaviour can be explained by the presence of hydrophilic groups, such as hydroxyl, amino and carboxyl groups that can be easily hydrated, both in chitosan and agarose [45, 46].

5.4.2. Evaluation of cell viability and proliferation in the presence of CAH

In vitro studies were performed using fibroblasts as model cells, since they are essential for the wound healing process to occur. These cells are involved in collagen, fibronectin, and synthesis of other biomolecules, that are structural components of the ECM, that provide a 3D support for the closure of tissue gaps and allow the restoration of the mechanical strength of the skin [47].

Figure S2 shows that cells adhered and remained viable in contact with the hydrogel and in the negative control (K^-), where cell spreading and elongation was visualized. Such process is comprised of a cascade of four different partly overlapping events: cell attachment, cell spreading, organization of actin cytoskeleton, and formation of focal adhesions [48]. The cellular response or interaction with the 3D polymeric matrices is mediated by integrins that recognize specific motifs at materials surface and allow physical anchoring as well as in signal transduction mechanisms that occur at the cell membrane [48, 49]. In the positive control (K^+), no cell adhesion or proliferation was observed. Dead cells with their typical spherical shape were observed.

To further characterize cell adhesion to materials surface a SEM analysis was also performed (see figure 5.3A). Fibroblasts attached and spread across the surface, presenting a round shape configuration, with some cytoplasm extensions towards the substrate after 24h of being seeded on CAH surface. After 72h, cells already presented the typical fibroblastic morphology,

lamellipodia connecting to surrounding fibroblast were visualized and a continuous layer of cells started to be formed [29].

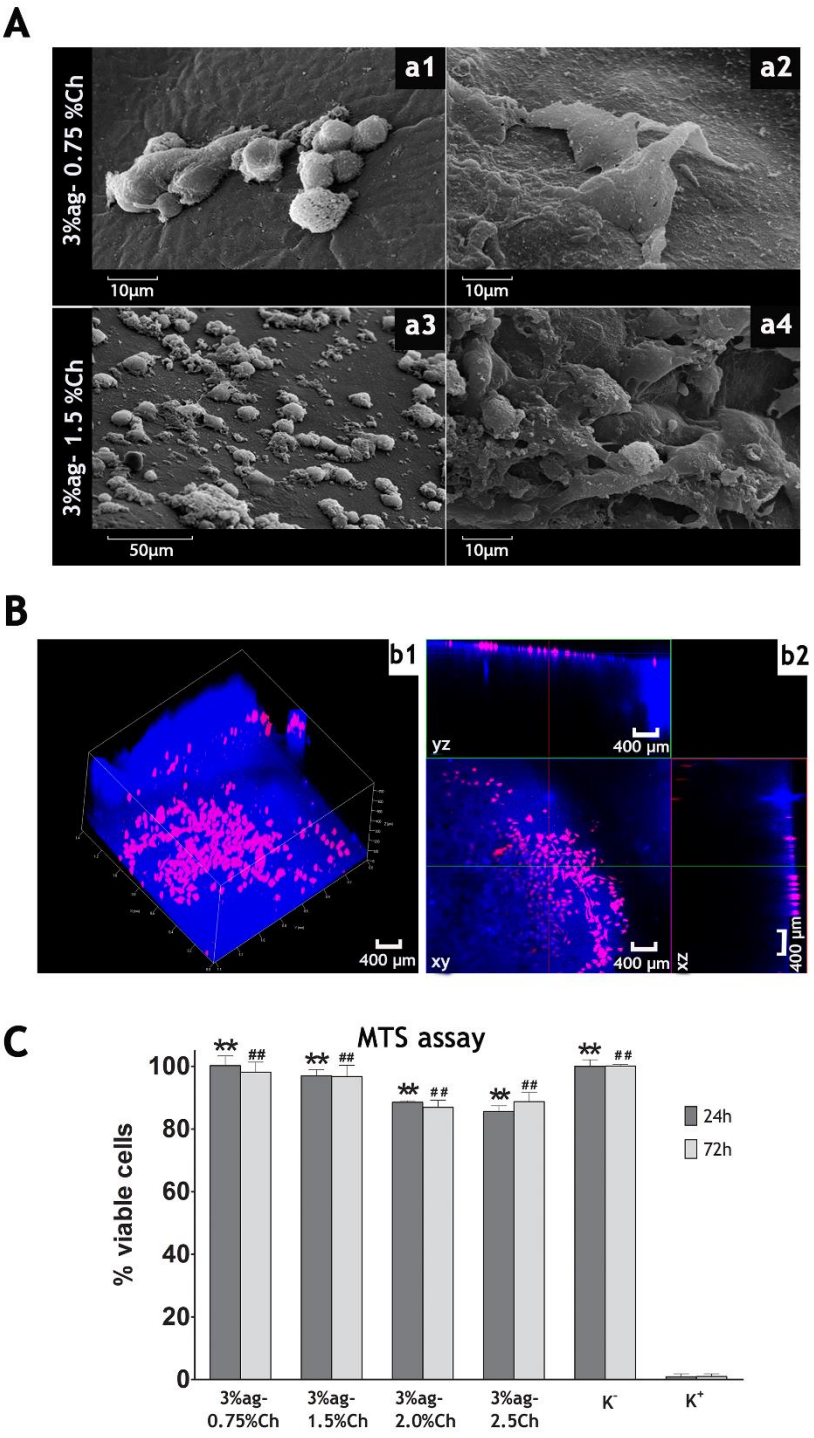

Figure 5.3: Evaluation of cell behavior in contact with hydrogel: (A) SEM images of NHDF in contact with: CAH (0.75% Ch) after 24 h (a1) and 72h (a2): CAH (1.5% Ch) after 24 h (a3) and 72h (a4); (B) 3D reconstruction of the cell internalization in the CAH (1.5% Ch) using stacked CLSM images (b1); orthogonal projection of CAH (1.5% Ch)in xy, yz and xz plans (b2). (C) Evaluation of cell viability through an MTS assay after 24 and 72h. K^+(dead cells); K^-(live cells); Each result is the mean ± standard error of the mean of at least three independent experiments. Statistical analysis was performed using one-way ANOVA with Dunnett' post hoc test and Newman-Keul smultiple comparison test (**,##,‡‡$p < 0.01$).

The cell behavior observed can be explained by the interaction of glycosaminoglycans, present at cell membrane, with the amine groups of chitosan present in CAH [29, 50]. Moreover, chitosan provides a matrix for 3D tissue growth and activates macrophages at the wound site that stimulate cell proliferation and histoarchitectural tissue organization [51].
In addition, fibroblast cells migration and proliferation within CAH was also characterized by confocal microscopy analysis. The CLSM images presented in figure 5.3B show that cells were able to migrate to the inner structure of CAH and the hydrogel pores were sufficiently large (90-400 μm) to accommodate NHDF, after 24h of culture, and also allow an effective nutrient supply and metabolic waste removal, processes that are essential for effective cell growth and subsequently skin regeneration [41, 52]. Cellular internalization is further noticeable in the orthogonal projection shown in figure 5.3b2.

5.4.3. Characterization of the cytotoxic profile of CAH

The materials cytocompatibility was also characterized through an MTS assay. The results presented in figure 5.3C show that the fibroblast cells viability was not affected, after 72h of being in contact with CAH, highlighting the biocompatibility of the produced hydrogel. A statistically significant difference was noticed between the positive ($p<0.01$) and the negative control and cells in contact with CAH. Moreover, the degradation by-products that could be produced during the 72h did not affect cell viability, which is also important to allow the application of the hydrogel for the aimed biomedical application.

5.4.4. Evaluation of the antimicrobial activity of the CAH

In this study, the CAH antibacterial properties were evaluated by standard tube dilution method using *S. aureus*. The bacterial strain was deemed appropriate for performing this assay, since it is reported in the literature as the most common gram-positive pathogen found in skin infections, when biomaterials are used for wound treatments [53].
Figure 5.4A shows *S. aureus* incubated with several formulations of hydrogel, containing different concentrations of chitosan (ranging from 125-400 μg/mL), during 24h at 37 °C. Afterwards, the CAH MIC values ranging between 188 - 200 μg/mL were determined (Figure 5.4-a2) using a standard procedure, a resazurin assay, described in literature [54]. The results obtained revealed a significant difference between the test groups and the positive control ($p<0.01$). CAH samples containing chitosan concentrations higher than 188 μg/mL exhibited antimicrobial activity, providing a defence barrier against the strain studied here. The polycationic chitosan, used in hydrogel production, can interact with the electronegative residues present at bacteria surface, increase the cell wall permeability and consequently the leakage of intracellular constituents and the dissipation of ionic gradients within bacteria [55, 56].

The bacterial biofilm formation at the biomaterials surface is one of the main causes of implant rejection [57]. These bacterial contaminations have the ability to evade host immune system response and also be resistant to different antibiotics [57]. Biofilm formation at CAH surface was also evaluated through SEM analysis. *S.aureus* was added to different formulations of CAH, during 24h as shown in figure 5.4(b1-b2). The results attained revealed that biofilm formation only occurred in the negative control (figure 5.4b3), where *S.aureus* was placed in agar plate without adding any antibacterial agent.

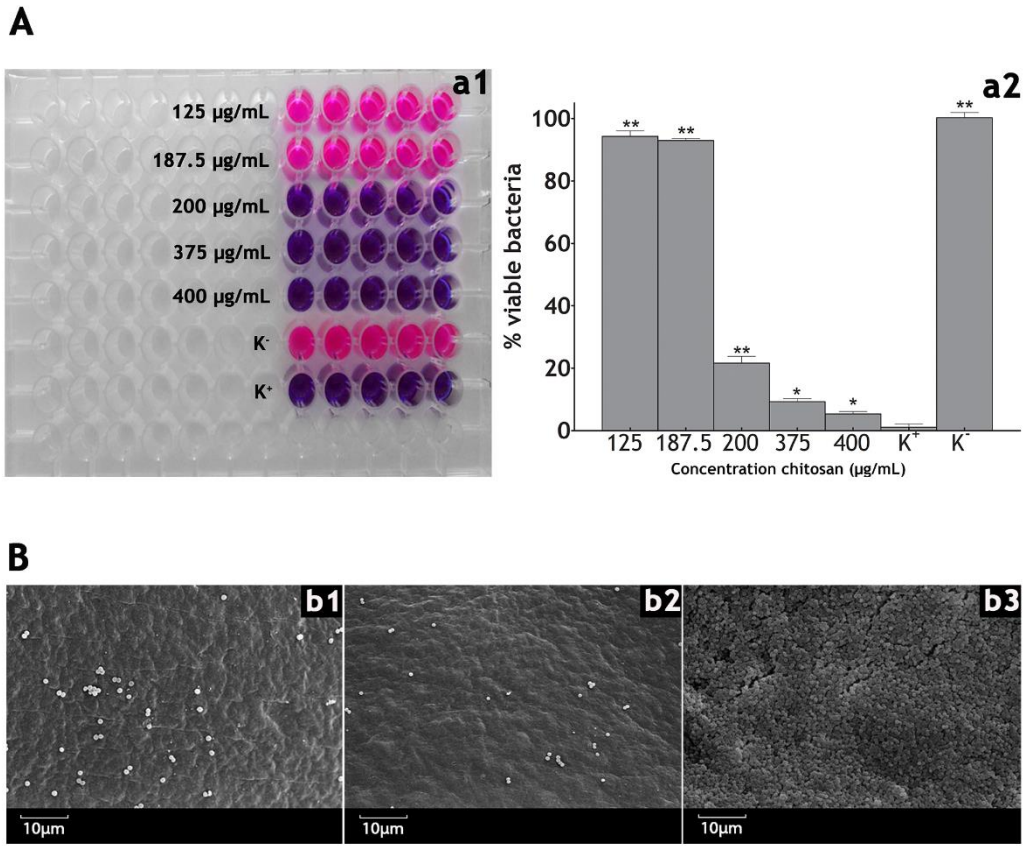

Figure 5.4: Characterization of antibacterial properties of hydrogel: (A) Determination of MIC values through a Resazurin assay (a1). Blue colour corresponds to the death bacteria and pink to the live bacteria. MIC values of the CAH after 24 h of incubation are presented in (a2). K^-(live bacteria); K^+(death bacteria); agarose (ag); chitosan (Ch). Each result is the mean ± standard error of the mean of at least three independent experiments. Statistical analysis was performed using one-way ANOVA with Dunnett's post hoc test (*p < 0.05;**p < 0.01); (B) SEM images of Staphylococcus aureus in contact with CAH produced with 200 µg/mL (b1) and 400 µg/mL (b2) of Ch after 24h. Negative control is presented in b3.

5.4.5. In vivo assays

The local and systemic histocompatibility of CAH was evaluated *in vivo*, by inducing transcutaneous full-thickness dermal wounds in Wistar rats. A minimum number of animals was used, taking into account the international guidelines set for animal experimentation. Wistar rats were initially divided into two groups. Group 1 was set as control, and wounds were only treated with serum physiologic solution. Group 2, test group, was used to study the influence

of CAH (3% agarose - 1.5% Chitosan), that was chosen based on the results previously achieved *in vitro*, in the wound healing process. All animals showed good general health condition throughout the study, as assessed by their weight gain. CAH was applied directly at the wound site, at a temperature of 40 ºC and gelled *in situ* at body temperature (37 ºC). Figure S3-A shows a set of typical wound beds after the surgical procedure and the different treatments to be applied (serum physiologic solution and CAH). The healing patterns were observed and wounds size measured for 21 days (figure S3-B). The wound size of animals from group 1 increased during the first days, while for the other group did not. Such result emphasizes the importance of an initial covering of the damage area with hydrogels, since they are usually composed by about 95% of water, gathering a moist environment that promotes wound re-epithelization by triggering epidermal cell migration at a speed of about 0.5 mm/day over a moist wound surface, which is twice as fast as that determined for dry wounds [58]. Furthermore, chitosan possesses biological activities that affect macrophage function, stimulates cell proliferation and contributes for histoarchitectural tissue organisation, as already described elsewhere [21, 59]. On the 21st day, the wounds of animals from Group 2 (treated with CAH) were completely healed (figure 5.5b4).

In vivo experiments showed that CAH can act as moist wound dressing due to its flexibility, permeability for water and metabolites, revealing its suitability for cleansing wounds by rehydrating dead tissues and enhancing autolytic debridement [9, 37, 60].

5.4.5.1. Histological analysis

The histological analysis of skin samples collected from the wounded site of animals from Group 1 showed that an acute inflammatory process occurred immediately after burn induction, as demonstrated by the presence of polymorphonuclear neutrophils (figure 5.5a1). Furthermore, after 21 days (figure 5.5a4), different inflammatory cells (granulocytes, macrophages and neutrophils) were also present at the wound. New capillary vessels and fibroblasts cells, involved in the production of collagen type III and other proteins from the ECM, were also observed. Although, skin full structural integrity was not completely restored.

Figure 5.5B presents the panorama of the wound healing process that occurred in animals treated with CAH (group 2). These animals presented a re-epithelization of the wounds completely different from that of non-treated wounds. Seven days after injury was induced (figure 5.5b1) no signs of acute inflammation were observed at the wound site.

Fibroblast cells were visible at the wound surface and granulation tissue was formed. On day 21 (figure 5.5b4), the area of the granulation tissue is higher than that presented by animals from group 1. Moreover, dermis formation and re-epithelization (the thickness of epidermis increased) occurred in group 2 but not in the control group.

The results obtained clearly demonstrate that the agarose/chitosan based hydrogel improves the wound healing process, and avoids bacterial infection at the wound site [61]. The thermoresponsive character of agarose is also fundamental to allow the gel to be applied directly at the desired site, without causing harmful side effects.

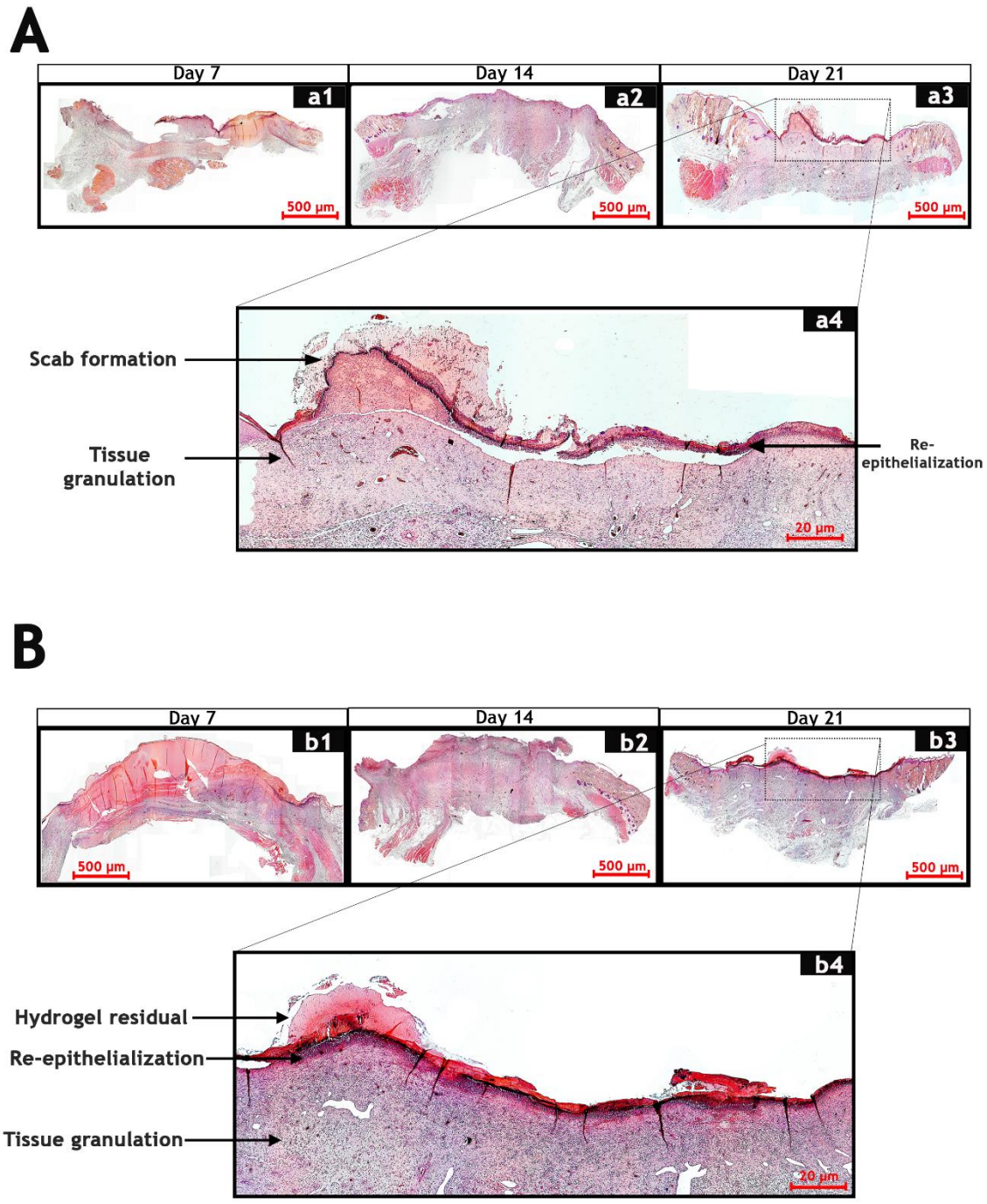

Figure 5.5: Hematoxylin and eosin-stained sections of collected tissue samples for morphological evaluation of wounds after 7, 14 and 21 days. Group 1 treated with saline solution (A) and group 2 treated with CAH (3% ag–1.5% Ch) (B). Agarose (ag); Chitosan (Ch).

5.5. Conclusions

Based on all data collected, the produced hydrogel provides an adequate wound-healing environment that fulfils the required criteria set forth for an "ideal" wound dressing. Specifically, the wounds covered with CAH were moist and hydrated, demonstrating that water loss and wound dehydration was prevented. The high cellular proliferation at hydrogels surface suggests that nutrients, oxygen and carbon dioxide exchange occurred and accomplish all demands within wound. No signs of bacterial infection have been observed at the wound site, emphasizing that CAH bacterial properties can avoid delays or absence of healing.
In a near future, the addition of other ECM components or growth and differentiation factors to the CAH will further improve the wound repair.

5.6. Acknowledgements

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Supplementary material from Chapter 5

Table S1: Degree of deacetylation of the different chitosan samples (mean ± SD, n=3).

Sample	Nominal DD[a] (%)	Determined DD[b] (%)
(1) Comercial Chitosan	75-85	83,35 ± 0,23
(2) Purified Chitosan	———	95,08 ± 0,48

[a] Provided by the manufacturer.

[b] Determined by 1DUVS.

Table S2: Physicochemical and biological characterization of the different hydrogels formulations used in this study.

Chitosan (%)	Agarose (%)	Tests						
		Angle water conctact	Swelling profile	SEM analysis	Confocal	Viability cell	Antimicrobial assay	In vivo assays
0.75	3							
1.5	3							
2.0	3							
2.5	3							

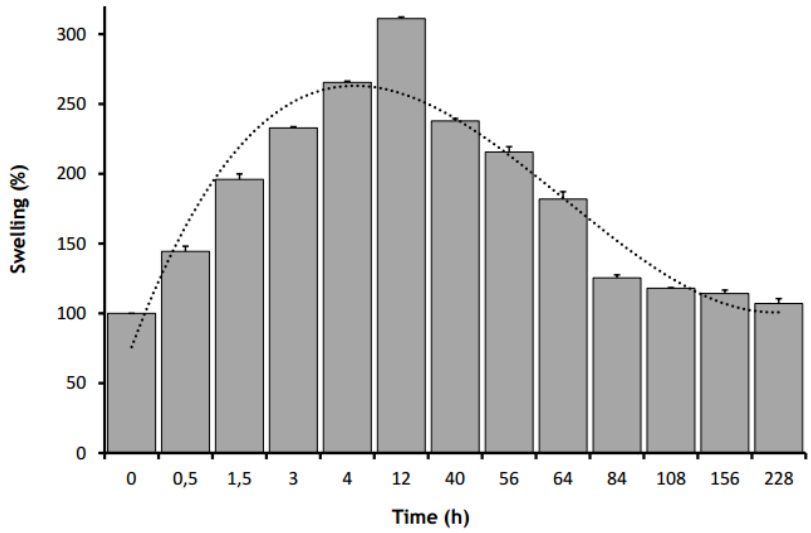

Figure S1: Swelling profile of the produced CAH along 228h.

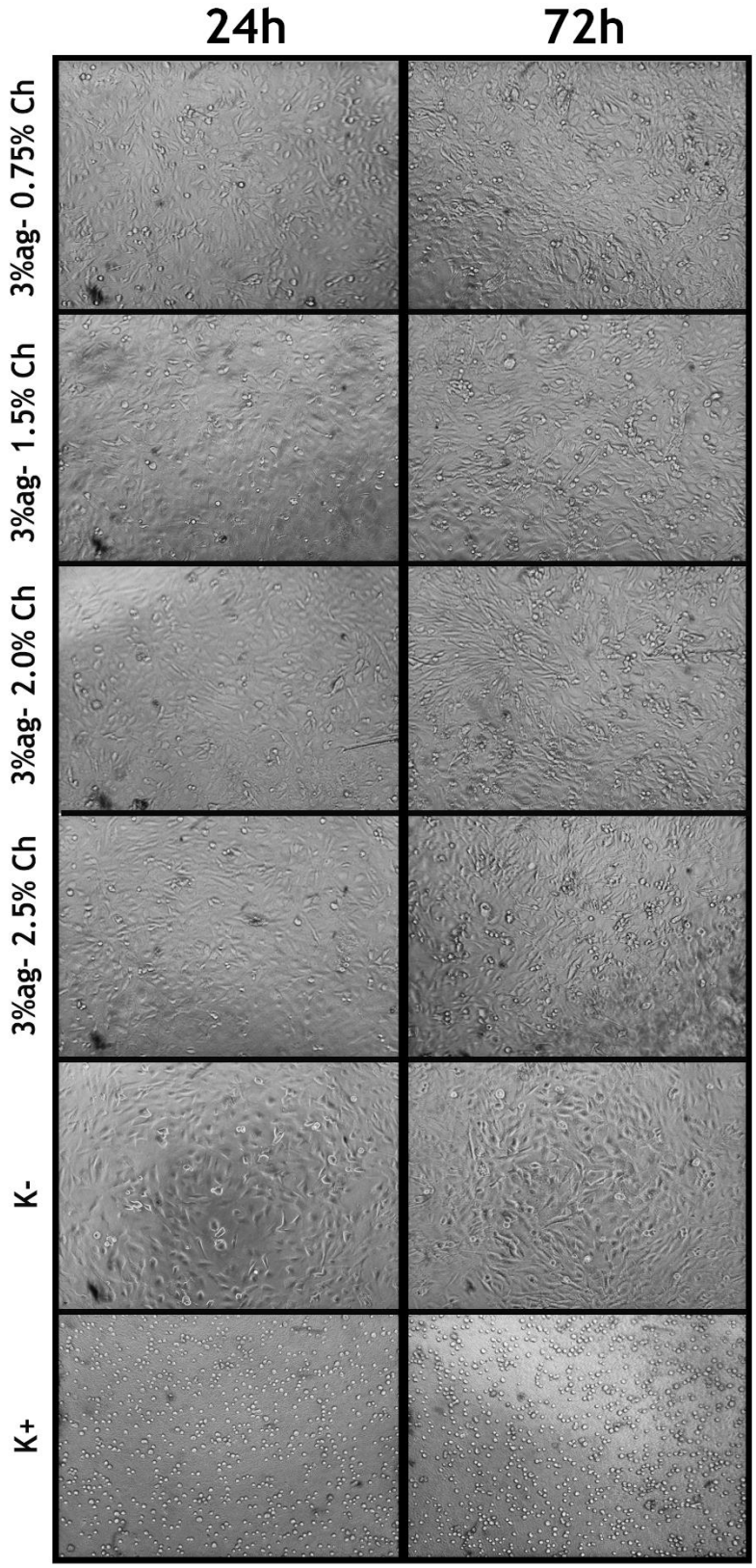

Figure S2: Microscopic images of human fibroblast cells after being seeded in the presence of the CAH during 24 and 72h; agarose (ag); Chitosan (Ch); negative control (K^-) (live cells); positive control (K^+) (dead cells). Original magnification 100x.

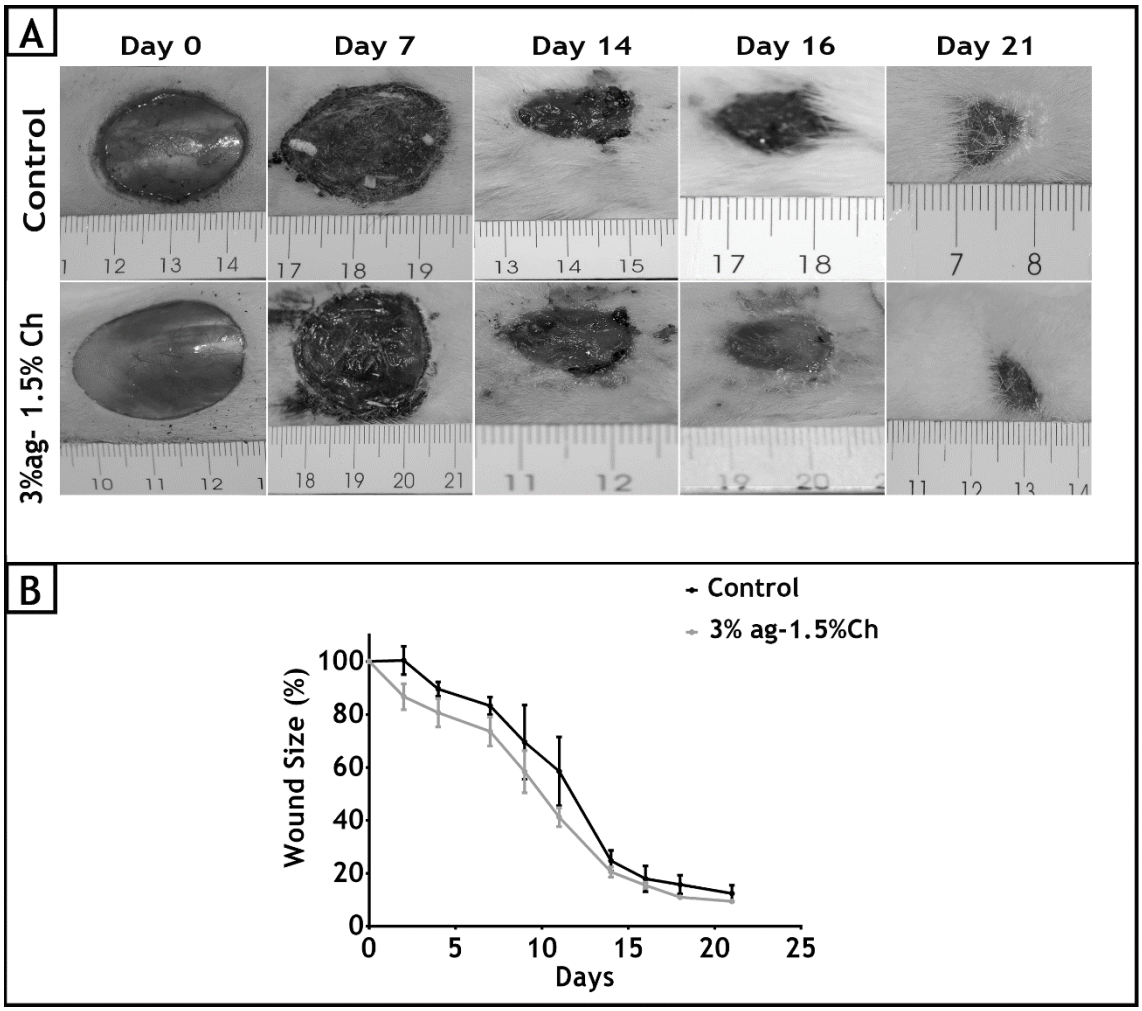

Figure S3: Macroscopic images of the wound-healing panorama over 21 days. A deep third-degree burn wound with 2 cm diameter was induced at the dorsal skin of each female Wistar rat. Digital images were acquired after the 7th, 14th, 16th and 21st days of injury be inferred (A). Effect of CAH (3% ag- 1.5 Ch) on burn wound compared to the control group. The surface area of the burn wounds was calculated as described in Methods section and reported at each time point as the percentage of the surface area at baseline. Each point represents the mean ± standard error of the mean of at least three independent experiments. Agarose (ag); Chitosan (Ch) (B).

Chapter 6
Concluding remarks and future trends

Concluding remarks and future trends

Every year, millions of patients suffer several skin lesions. These injuries need to be treated in order to restore the native properties of the skin and avoid permanent damage or even patient death. Over the past two decades extraordinary advances in cellular and molecular biology have greatly expanded the comprehension about the basic biologic processes involved in wound healing. Such knowledge led to advances in the treatment of different wounds. Nowadays, autograft is the best solution for the replacement of lost skin, however this graft is not always available, particularly when a large body surface area is damaged or when the patient health condition does not allow the harvesting of skin. Allografts and xenografts can be an alternative to the autografts, nevertheless problems associated with graft rejection, availability, cultural and ethical issues and disease transfer may occur.
In order to overcome these problems, different wound dressings have been developed and some of them are already used in the clinic.
However, none of the currently available dressings are capable of fully re-establish and reproduce skin properties, thus stressing the importance of developing new wound dressings with a broad applicability and efficiency. Recent studies highlighted the potential of hydrogels to be used in clinic, as skin substitutes, by combining the properties of polymers with bioactive compounds to improve the wound healing process.

The present research work aimed to develop new hydrogels using natural polysaccharides, such as chitosan, dextran and agarose to be used as skin substitutes for wound healing. Furthermore, vascular endothelial and ephetilial growth factors were loaded within microparticles that were subsequently incorporated in the hydrogel to further improve the wound healing process. Such allowed the release of growth factors with a suitable profile, according to the demands of the healing process.

In the first study (chapter 3) it was described the development of a chitosan hydrogel to be used as a skin substitute. In this study a chitosan solution was simply put in contact with gaseous ammonia to induce gelation. The results obtained revealed that the hydrogel is highly porous and has an interconnecting inner structure. Optical microscopic analysis showed cell growth and proliferation in the presence of the hydrogel, revealing hydrogel cytocompatibility. *In vivo* studies showed that this 3D polymeric structure did not induce an acute inflammatory response in the host and improved the wound healing.

The second article (chapter 4) reported the preparation/application of an *in situ* crosslinkable, biodegradable hydrogel of oxidized dextran loaded with chitosan microparticles containing vascular endothelial and epithelial growth factors. The *in vitro* assays revealed that this system is biocompatible. The *in vivo* assays suggested that the dextran hydrogel, containing chitosan

microparticles loaded with the two growth factors, promotes a faster wound healing with, no signs of local or systemic inflammatory responses. Furthermore, dextran hydrogel could be an excellent choice for the application on wounds with rough edges once the hydrogel is cross-linked *in situ*. Moreover, this system can be used in a near future for the controlled delivery of other bioactive molecules applied in tissue regeneration.

The work described on Chapter 5, presents a thermoresponsive hydrogel produced using two natural biocompatible polysaccharides, chitosan and agarose. The morphological analysis showed that the hydrogel has a porous structure, and the contact angle analysis revealed a moderate hydrophilic surface. Furthermore, cells were able to adhere and proliferate at the surface and within the hydrogel. *In vivo* studies demonstrated that the hydrogel improves the wound healing. No signs of bacterial infection were observed at wound site. Based on all data collected, the produced hydrogel showed to be a versatile skin substitute.

Overall this research showed the good performance of natural, bioavailable and cheaper polymers for the development of skin substitutes, and the relevance of the addition of external factors (growth factors), and its controlled release, during the process of wound healing. Moreover, the combination of the different polymers allowed the improvement of both mechanical and biological properties of the produced hydrogels.

In a near future, the addition of extracellular matrix components, growth and differentiation factors and nucleic acids to the produced hydrogels will be explored to characterize their role in wound repair. Moreover, cell encapsulation within these polymeric hydrogels can also be assayed for improving heal, body appearance and scar formation.
The application of these hydrogels can be extended to other research areas of Tissue Engineering like nervous system, cartilage, ocular lens or even organs production.