UNIVERSIDADE DA BEIRA INTERIOR

Ciências da Saúde

Development of a novel hydrogel for skin regeneration

Sónia Alexandra Pereira Miguel

Dissertação para obtenção do Grau de Mestre em

Ciências Biomédicas

(2° ciclo de estudos)

Orientador: Ilídio Joaquim Sobreira Correia, Ph.D.
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List of Publications

- Articles in peer reviewed international journals:

  Ribeiro, M., Morgado, P., Miguel, S., Coutinho, P.and Correia, I.; Dextran based-hydrogel containing chitosan microparticles loaded with growth factors to be used in wound healing. *Materials Science and Engineering: C*. (2013). **33** (5): 2958-2966.

- Poster communications:

  Patrícia I. Morgado, Maximiano P. Ribeiro, Sónia P. Miguel, Ilídio J. Correia, Ana Aguiar-Ricardo, Development of poly(vinyl alcohol) and chitosan wound dressings using supercritical fluids technology, 11º Encontro Nacional de Química-Física, 9 – 10th of May, Faculty of Sciences, University of Porto, Portugal.

- Oral presentations:

  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 Symposium on Technology and Health, Instituto Politécnico da Guarda (IPG), 3rd of May 2013, Guarda, Portugal.

"Para ser grande, sê inteiro; nada teu exagera ou exclui; sê todo em cada coisa; põe quanto és no mínimo que fazes; assim em cada lago, a lua toda brilha porque alta vive." **Fernando Pessoa**

Acknowledgments

At the end of this master thesis, there are a lot of people that I would like thank, by the support and courage. One way or another, they all contributed to this work has been done.

First, I would like to thank to my supervisor Professor Ilídio Correia for the unconditional support, guidance, patient, and help during my master's degree. Their wise advices help me to grow as an investigator and person. I also feel the need to acknowledge him for having believed in my capabilities and for making me believe in myself. It has been a privilege working with him.

I would like thank to my co-supervisor Maximiano Ribeiro, for all guidance and support in progress of my work. His friendship and advices were, without any doubt, very important to overcome the difficulties faced.

I also would like to thank to Eng. Ana Paula from the Optical Center of Universidade da Beira Interior for the help in acquiring scanning electron microscopy images of the hydrogel produced.

I would like to thank my entire colleagues group for their support, knowledge sharing, force and courage in moments less good, but also for all the moments of fun and happiness that we had together, without a doubt, I have been blessed with a friendly and cheerful group.

To my closest friends that have always accompanied me in these last 5 years. They always believed in me and they helped me to always have a smile in my face. "In difficult times, is in the hug of a friend that we find the strength to continue". Thank you all from the bottom of my heart.

Lastly, and most importantly, I would also like to express my sincere thanks to my parents, António and Helena and my sister, Telma. Thanks for the unconditional support and for always believing in me when I doubted about myself. They are truly important in all of my victories; your love is enough to make me feel stronger. To them I dedicate this thesis.

To all of you… a thank you…

Abstract

Skin lesions are traumatic events that lead to increased fluid loss, infections, scars formation and the appearance of immunocompromised regions. The loss of skin integrity can result in significant physiological imbalances and disability or even death. Skin functionality must be restored quickly in order to maintain homeostasis. Researchers have been developing new systems to accelerate the healing process. Although the many skin substitutes available in the market, there is none that promotes full restoration of the native structure of skin. Among the various materials used to cover the wound immediately after injury, hydrogels are the most studied. The hydrogels are made of a highly hydrophilic polymeric network forming a three dimensional structure very similar to the extracellular matrix, that allows cell growth. Moreover, hydrogels are biocompatible, biodegradable and have porous structures that allow cell internalization and proliferation within its structure and promote the diffusion of gases, nutrients and waste products. Currently, new hydrogels that respond to external stimuli such as pH, and temperature have been extensively studied in tissue engineering. Thus, the work plan of this master thesis had as main goal to produce a hydrogel composed of deacetylated chitosan and agarose, formed at body temperature (37°C), in order to verify their applicability in the treatment of wounds. The hydrogel structure was initially characterized by Fourier transform infrared spectroscopy. Its inner and surface morphology was characterized by scanning electron microscopy. Cellular adhesion and internalization into the porous structure of the hydrogel was visualized by confocal and scanning electron microscopy. The cytotoxicity profile of the hydrogel was characterized through cell viability assays, and the results obtained confirmed the biocompatibility of the hydrogel. The antimicrobial activity of the hydrogel was also evaluated and the results showed that the hydrogel inhibits the growth, at the surface, of the most common microorganism in skin infection. The results obtained demonstrated that this 3D network has the suitable properties for improving the healing process of cutaneous wounds.

Keywords

Antimicrobial activity, Biocompatibility, Cell internalization, *In vitro studies*, Wound healing.

Resumo

As lesões na pele são acontecimentos traumáticos que levam ao aumento da perda de fluidos, a infecções, à formação de cicatrizes e ao aparecimento de regiões imunocomprometidas. A perda da integridade da pele pode resultar em desequilíbrios fisiológicos e incapacidade significante ou mesmo até a morte do paciente. A funcionalidade da pele deve ser restaurada rapidamente, de forma a manter a homeostase. Os investigadores têm procurado desenvolver novos substitutos de pele que permitam acelerar o processo de cicatrização. Apesar de já existirem muitos substitutos de pele disponíveis no mercado, ainda não existe nenhum que promova o restabelecimento da estrutura nativa da pele, na sua totalidade. Entre os mais variados materiais utilizados para cobrir a ferida, imediatamente após a lesão, os hidrogéis são a classe de materiais mais estudada. Os hidrogéis são constituídos por uma rede polimérica altamente hidrofílica, que forma uma estrutura tridimensional muito semelhante à matriz extracelular. Os hidrogéis promovem a adesão e o crescimento celular. Normalmente, os hidrogéis apresentam estruturas porosas que possibilitam a internalização e proliferação das células no seu interior, a difusão de gases, nutrientes e resíduos. Alem disso, os hidrogéis são biocompatíveis e biodegradáveis. Atualmente, os hidrogéis que respondem a estímulos externos como o pH, temperatura têm sido amplamente estudados na engenharia de tecidos. Assim, o plano de trabalhos deste mestrado teve como objetivo a produção de um hidrogel constituído por quitosano desacetilado e agarose, o qual se forma à temperatura corporal (37°C), para ser aplicado no tratamento de feridas cutâneas. A composição química do hidrogel foi analisada por espectroscopia de infravermelho com transformada de Fourier e a morfologia da superfície e do interior foi caraterizada por microscopia electrónica de varrimento. A visualização da adesão e internalização celular no hidrogel foi conseguida através de imagens de microscopia electrónica de varrimento e confocal. O perfil citotóxico do hidrogel foi caraterizado através de testes de viabilidade celular, os quais confirmaram a biocompatibilidade do hidrogel. A atividade antimicrobiana do hidrogel foi também avaliada e os resultados obtidos confirmaram que o hidrogel inibe à sua superfície, o crescimento do microorganismo mais comum em infecções cutâneas (*Staphylococcus aureus*). Os resultados obtidos neste trabalho demonstram que o hidrogel desenvolvido possui as propriedades adequadas para ser usado na regeneração das feridas cutâneas.

Palavras-chave

Actividade antimicrobiana, Biocompatibilidade, Cicatrização de feridas, Estudos *in vitro,* Hidrogel, Internalização celular.

Resumo alargado

A pele é o maior órgão do corpo humano, podendo pesar entre 6 a 10 kg. Em adultos, apresenta uma área aproximada de $2m^2$. Está envolvida na preservação da homeostase dos fluidos do corpo, na regulação térmica e na protecção contra agentes infecciosos. Diariamente, a pele está sujeita à perda da sua integridade devido a uma doença ou trauma. As lesões na pele são descritas como resultado da disrupção da estrutura anatómica e funcional da pele. As lesões cutâneas podem ser causadas por uma interrupção precisa de tecido pelo bisturi do cirurgião (incisão), por grandes danos no tecido (por exemplo, trauma, queimaduras), ou ainda resultado de uma contusão, hematoma, laceração ou uma resistência à abrasão. Consoante, o tipo e duração do processo de cicatrização das feridas, estas podem ser classificadas como agudas ou crónicas e que podem resultar num desequilíbrio fisiológico, numa deficiência significativa ou até mesmo na morte do indivíduo.

Desta forma, a funcionalidade da pele deve ser restaurada rapidamente, de forma a manter a sua homeostase. A cicatrização de feridas é um processo dinâmico e complexo que inclui uma série de fases como, coagulação, inflamação, síntese e deposição da matriz extracelular, angiogénese, fibroplasia, epitelização, contração e remodelação. Estas fases do processo cicatricial ocorrem em cascata e envolvem a migração de vários tipos de células para o local da lesão. Após uma lesão, a pele necessita ser revestida de forma a diminuir a dor, a contaminação, o risco de infeção e, por outro lado promover o restabelecimento da integridade da pele. Esta necessidade fez com que nas últimas décadas um grande número de substitutos de pele fosse desenvolvido e introduzido no mercado. Os substitutos de pele, estabelecem uma barreira mecânica contra microorganismos e previnem a desidratação; promovem a entrega de componentes da matriz extracelular (colagénio, proteínas de adesão), citocinas e fatores de crescimento que contribuem para melhorar o processo de cicatrização normal; e ainda, fornecem uma estrutura de suporte para incorporação de células e moléculas bioativas, as quais são aplicadas no local da lesão e auxiliam o processo de regeneração.

Muitos substitutos de pele têm sido aplicados no tratamento de feridas cutâneas, como é o caso dos auto-, alo- e xeno- enxertos. No entanto, estas abordagens terapêuticas apresentam inúmeras limitações tais como, a rejeição por parte do paciente, o risco de transmissão de doenças e, ainda a disponibilidade limitada dos recursos em relação à procura. De modo a suplantar estas limitações, investigadores têm procurado desenvolver novos substitutos de pele à base de biomateriais que permitam acelerar o processo de cicatrização. Existem já substitutos de pele disponíveis no mercado, classificados de acordo com a camada da pele que pretendem substituir (Epidérmicos, Dérmicos e Dermo-epidérmicos), os quais são constituídos à base de polímeros naturais/ sintéticos e, alguns deles possuem células encapsuladas (fibroblastos e/ou queratinócitos).

Entre os mais variados tipos de materiais utilizados para a produção de uma estrutura que promova a cicatrização de uma ferida cutânea, os hidrogéis surgem como os substitutos mais estudados na engenharia de tecidos devido ao seu caráter hidrofílico, que forma uma rede tridimensional muito semelhante à matriz extracelular. O seu alto conteúdo em água dos hidrogéis torna-os biocompatíveis para a maioria dos tecidos vivos e a sua natureza viscoelástica diminui o dano do tecido circundante, quando implantado no hospedeiro.

Os hidrogéis fornecem um microambiente que contém poros suficientemente grandes para permitir a adaptação das células, as quais podem entrar e proliferar no interior da rede polimérica. Adicionalmente, estes poros permitem também, a difusão de gases, nutrientes e resíduos. Polímeros naturais como agarose e o quitosano são dos mais usados na produção de hidrogéis pois não induzem uma resposta imunológica crónica no hospedeiro, uma vez que estes são biodegradáveis e biocompatíveis.

Os métodos tradicionais usados para a produção de hidrogéis incluem a co-polimerização e a reticulação dos percursores poliméricos. Estes métodos de síntese de hidrogéis não permitem um controlo preciso da sua estrutura, produzindo hidrogéis com deficientes propriedades mecânicas. Por conseguinte, os hidrogéis que respondem a estímulos externos como o pH e temperatura têm sido amplamente estudados na engenharia de tecidos. Hidrogéis que são produzidos apenas pela alteração da temperatura, no local da lesão oferecem diversas vantagens, uma vez que, não necessitam de solventes orgânicos ou agentes de co-polimerização para reticular, permitem que o processo seja mais simples e rápido. Além disso, o hidrogel que é polimerizado na lesão torna o método menos invasivo, aumenta a viabilidade e melhora o conforto ao paciente, reduzindo-lhe a dor associada ao processo de cicatrização de feridas. No entanto, nenhum destes substitutos de pele promove o restabelecimento da estrutura nativa da pele, na sua totalidade.

Assim, o plano de trabalhos deste mestrado teve como objetivo avaliar a aplicabilidade de um hidrogel, constituído por quitosano desacetilado e agarose no tratamento de lesões de pele. A composição química do hidrogel foi analisada por espectroscopia de infravermelho com transformada de Fourier e a morfologia do hidrogel foi caracterizada através de imagens de microscopia electrónica de varrimento e microscopia confocal. A biocompatibilidade deste biomaterial foi avaliada através de ensaios *in vitro*. Fibroblastos humanos, foram usados como modelos nos ensaios *in vitro,* de modo a avaliar a citotoxicidade do hidrogel. A atividade antimicrobiana do hidrogel foi também avaliada, através de ensaios microbiológicos com *Staphylococcus aureus*. Verificou-se que agarose e quitosano desacetilado estabeleciam pontes de hidrogénio entre si e formavam um hidrogel poroso. Os fibroblastos aderiram e proliferaram na presença do hidrogel em estudo, o que permitiu concluir a ausência de citotoxicidade deste, assim como dos produtos resultantes da sua degradação. Os resultados obtidos dos ensaios de microbiologia confirmaram que o hidrogel inibe à sua superfície, o crescimento do *Staphylococcus aureus*. Os resultados obtidos sugerem que o hidrogel desenvolvido possui as propriedades adequadas para ser usado na regeneração das feridas cutâneas.

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List of Abbreviations

3D	Three-dimensional
Auto	Autologous
BSA	Bovine Serum Albumin
CAH	Chitosan-Agarose Hydrogel
CFU	Colony-Forming Unit
CSLM	Confocal Laser Scanning Microscopy
DD	Degree of Deacetylation
DEJ	Dermal-Epidermal Junction
DMEM	Dulbecco's Modified Eagle's Médium
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
EtOH	Ethanol
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
FTIR	Fourier-Transform Infrared Spectroscopic
GAGs	Glycosaminoglycans
HA	Hyaluronic Acid
K^-	Negative Control
K^+	Positive Control
MIC	Minimum Inhibitory Concentration
MMP	Metalloproteinases
MMW	Medium Molecular Weight
MTS	(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
P(PF-co-EG)	Poly (Propylene Fumarate- co- Ethylene Glycol)
PAA	Poly (Acrylic Acid)
PBS	Phosphate Buffered Saline

PDFs	Platelet-Derived Growth Factors
PEO	Poly (Ethylene Oxide)
PGA	Polyglycolic Acid
PI	Propidium Iodide
PLA	Polylactic Acid
PVA	Poly (Vinyl Alcohol)
RGD	Arginine–Glycine–Aspartic
SEM	Scan Electron Microscopy
Synth	Synthetic
TE	Tissue Engineering
TGF	Transforming Growth Factors
TIMP	Tissue Inhibitors of Metalloproteinases
US	United States
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organization

Chapter I

Introduction

1. Introduction

The skin is the largest organ of the human body and plays highly specialized functions. It protects against toxins and microorganisms from the environment and prevents the body from dehydration. It is also involved in other functions, such as immune surveillance, sensory detection, and self-healing. Overall, skin is an indicator of general well-being and health state of each individual (Holbrook and Wolff, 1993, Clark *et al.*, 2007, Menon and Kligman, 2009, Lanza *et al.*, 2007).

Skin injuries have a high impact on the life quality of patients. In fact, the World Health Organization (WHO) estimates that, annually, over 300 000 millions suffer from a partly devastating physical and emotional consequences thereof (Yildirimer *et al.*, 2012). Skin injuries are caused by burns, accidents and diseases. Depending on the severity of the wound, loss of skin integrity may result in substantial physiologic imbalance and ultimately in significant disability or even patient death (Zhang and Michniak-Kohn, 2012).

The most common cause of significant skin loss is thermal injury, which accounts for approximately one million of emergency visits per year to the health care system, just in the United States (US) (Association, 2005). Depending on the location and depth of the burn, a victim may experience a wide number of potentially fatal complications including shock, infection, electrolyte imbalances and respiratory failure. Beyond physical complications, burns can also result in severe psychological and emotional distress due to long-term hospitalization, scarring and deformity (Evers *et al.*, 2010). For a complete restoring of both skin structure and function, successful wound healing must occur. The healing process of an adult skin is complex, requiring the collaborative efforts of several tissues and cell lineages, as well as both extracellular and intracellular signals (Kirker *et al.*, 2002, Gurtner *et al.*, 2008, Sun *et al.*, 2011).

In order to solve the problems associated with re-establishment of skin native structure and to promote the mechanisms responsible for improving the healing, some research groups have investigated the development of wound dressings that could protect the wound from bacterial infection, dehydration and absorption of the wound exudate, in order to enhance the healing process (Kirker *et al.*, 2002). Skin substitutes have a high demand for clinical uses. Actually, they represent approximately 50% of tissue engineering and regenerative medicine market revenues. By the year of 2019, the total potential target population for the use of tissue-engineered skin replacements and substitutes is expected to increase to 6.4 million, resulting in a potential market just in US of approximately 24.3 billion dollars (Zhang and Michniak-Kohn, 2012).

In a near future, Tissue Engineering (TE) purposes to produce a biodegradable wound dressing that promotes the re-establishment of skin's native structure (epidermis, dermis and skin

appendages). Furthermore, these skin substitutes are expected to reduce costs and pain associated with skin regeneration (Lanza *et al.*, 2007).

The introduction section of this MSc thesis aims to describe the problems and strategies that are currently being developed to improve skin regeneration. The others sections describe the methods, results and conclusions obtained in this study.

1.1 Skin

1.1.1 Functions and structure

Skin occupies almost $2m^2$ of surface area and accounts for 8% of the body's mass (Hunt *et al.*, 2009, Bhat and Kumar, 2012, Marieb and Hoehn, 2010). Skin is an anatomical barrier between the organism and environment, which aims to protect it from toxic substances, pathogens and microorganisms (Hunt *et al.*, 2009, Zhang and Michniak-Kohn, 2012, Holbrook and Wolff, 1993). Essentially, skin is involved in four main functions, such as (Young, 2006, Hunt *et al.*, 2009, Marieb and Hoehn, 2010).

- **Protection**: the skin provides protection against ultraviolet light, mechanical, chemical and thermal insults. Moreover, its relatively impermeable surface prevents patient dehydration and acts as a physical barrier to avoid microorganisms invasion.

- **Sensation:** the skin is largest sensory organ in the body and contains a variety of receptors for touch (Meissner's Corpuscle), pressure (Pacini Corpuscle), pain (nociceptors) and temperature (thermo-receptors).

- **Thermoregulation:** in humans, skin is a major organ of thermoregulation. The body is insulated against heat loss by presence of hairs and subcutaneous adipose tissue. Heat loss is facilitated by evaporation of sweat from the skin surface and increased blood flow through the rich vascular network of the dermis.

- **Metabolic functions:** subcutaneous adipose tissue constitutes a major store of energy, mainly in the form of triglycerides. Vitamin D, which is responsible by maintenance of calcium and phosphorus concentration in blood, is synthesised in the epidermis.

Human skin also provides a potential path for the transport of functional active therapeutic substances such as drugs/reagents/biomolecules into blood stream (topical delivery) and/or the body (transdermal delivery) (Guy and Hadgraft, 2003). Skin is structurally complex and highly specialized consisting of two intimately associated layers known as the epidermis and dermis (Lodish *et al.*, 2001, Hunt *et al.*, 2009). Some authors refer a third layer, the hypodermis or subcutaneous layer, that is mainly composed of fat (Metcalfe and Ferguson, 2007a). The epidermis is mainly responsible for providing a barrier to prevent water loss and infection (Elias, 2007). Epidermis and dermis are separated by the extracellular matrix (ECM) also known as the basal lamina (Ajani *et al.*, 2007, Horch *et al.*, 2007). Certain skin appendages such as hair follicles and sweat glands, span from the epidermis to dermis and penetrate into the subcutaneous adipose tissue below the dermis, also known as hypodermis (Lodish *et al.*, 2000, Metcalfe and Ferguson, 2007b). The main functions of the hypodermis comprises the providing of blood vessels and nerves to the skin and also allow its connection to the underlying bones and muscles (Seeley *et al.*, 2005).

So, three main cell populations are found in the skin: epithelial cells covering the surface as the epidermis, but also forming the structures of the hair follicles, sweat and sebaceous glands; the fibroblasts, which are found in the dermis, give shape and maintain skin structure; and the endothelial cells that are found in the blood vessels (Shakespeare, 2001).

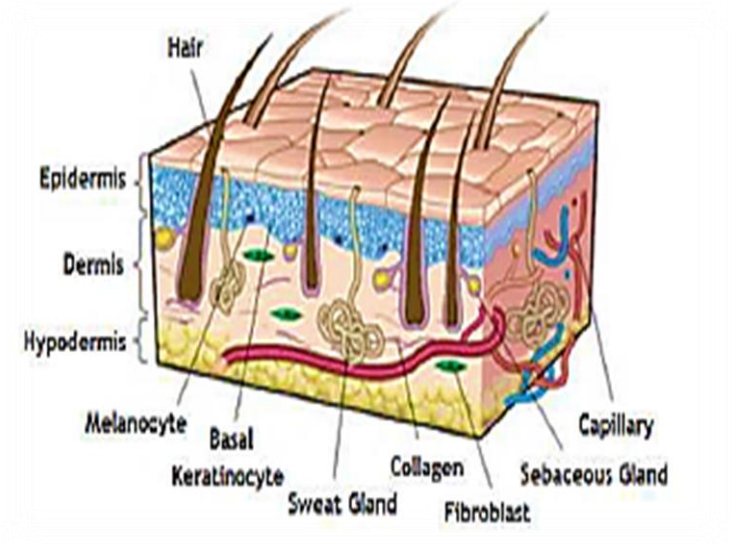


Figure 1 :Scheme of skin structure (adapted from(Gurtner *et al.*, 2008)).

1.1.2 Epidermis

The outermost layer of the skin, known as the epidermis, is mainly responsible for providing a barrier function to prevent water loss and infections (Brohem *et al.*, 2011). The epidermis is mainly constituted by keratinocytes (90-95%), which proliferate in the *stratum basale* and are responsible for the resistance as well as the structural characteristics and impermeability of the skin (Seeley *et al.*, 2005, Scheuplein, 2011). Notwithstanding, this layer also contains other cell types such as Langerhans cells, which have an essential role in the skin immunological defense (Regnier *et al.*, 1998), melanocytes that contribute for the color of the skin and Merkel cells, that are thought to play a sensory role (Boyce and Warden, 2002). The epidermis does not contain any blood vessels and, as a result, it is usually possible to rub off dead cells from the epidermis without bleeding (Marieb and Hoehn, 2010, Hunt *et al.*, 2009). The continuous process of all proliferation, differentiation and ultimately, death and shedding, allow the compartmentalization of this layer of skin into a number of sub- layers representing different stages of keratinocyte maturation.

Cells produced by proliferation of the germinal basal layer adjacent to the dermis undergo maturational changes related with the production of keratin. The outer keratinised layer is removed continuously and is replaced by the progressive movement and maturation of cells from the germinal layer. The rate of the mitosis in the germinal layer generally equals the rate of desquamation from the outer surface. As shown in Figure 2, the stages of this dynamic process are represented on four morphological sub-layers. The *stratum basale* is the germinal layer of the epidermis and its mitotic activity provides a constant supply of new keratinocytes to replace those lost by normal wear and tear. The *stratum spinosum* contains cells that are in the process of growth and early keratin synthesis. The *stratum granulosum* is characterized by intracellular granules that are involved in the process of keratinisation. The *stratum corneum* consists of flattened, fused cell remnants are mainly composed by fibrous protein, keratin (Zhang and Michniak-Kohn, 2012, Young, 2006, Scheuplein, 2011, Balasubramani *et al.*, 2001).

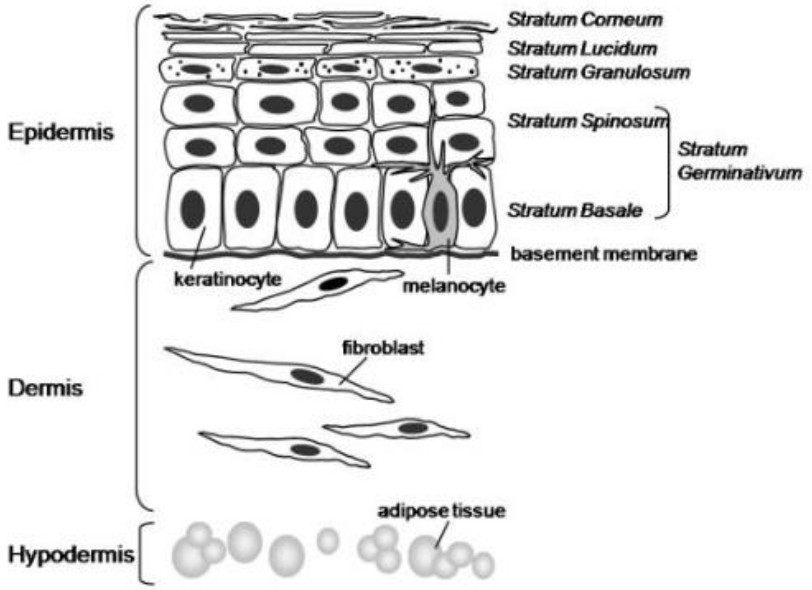


Figure 2: Representation of the skin components and layers. Epidermis contains melanocytes and keratinocytes that are able to differentiate and form the different strata (*corneum*, *luccidum*, *granulosum*, *spinosum* and *basale*). Dermis contains fibroblasts embedded in a matrix. Hypodermis is composed by the adipose tissue (adapted from (Brohem *et al.*, 2011)).

The epidermis is bound tightly to the underlying dermis through the basement membrane at the Dermal-epidermal junction (DEJ). The basement membrane can be divided into *lamina lucida* (the layer closer to the epidermis that is made of laminin, integrins, entactins, and dystroglycans) and *lamina densa* (a sheet-like structure composed mainly collagen type IV). The whole basement membrane is involved in the mechanically stabilization of the epidermis (Zhang and Michniak-Kohn, 2012, Seeley *et al.*, 2005, Inoue *et al.*, 2005).

1.1.3 Dermis

The dermis lies below the epidermis and constitutes the main part of the skin (Brohem *et al.*, 2011). The cellular components of dermis include fibroblasts (as shown in Figure 2), endothelial cells, smooth muscle cells, macrophages and adipocytes. Fibroblasts are responsible for the production of collagen, elastic fibers, glycosaminoglycan and glycoproteins, that comprise the ECM (Zhang and Michniak-Kohn, 2012, Seeley *et al.*, 2005). The expression and function of these ECM proteins and their receptors are highly regulated spatially and temporally during wound healing and tissue remodelling. Inappropriate deposition of ECM components will impair the normal healing and function of this tissue. The ECM components play important roles in every step of wound healing processes by providing

both scaffold support and playing signaling roles, promoting cell adhesion and migration during wound repair. Furthermore, they also mediate interactions between cells and matrix and act as reservoir and modulator for growth factors. Such is fundamental for connecting epidermis to dermis and also for restoring the integrity and function of the skin (Hunt *et al.*, 2009, Seeley *et al.*, 2005, Li and Kirsner, 2005).

The dermis is composed by the *papillary* and the *reticular dermis*. The *papillary dermis* contains more cells than fibers. It also holds numerous blood vessels that provide nutrients to the overlying epidermis, remove waste products and even help to regulate the body temperature (Seeley *et al.*, 2005, Zhang and Michniak-Kohn, 2012). On the other hand, the *reticular* dermis is mainly composed by dense collagen and elastic fiber matrix, being the main source of strength and flexibility of the skin (Eckhart *et al.*, 2003, Inoue *et al.*, 2005, Seeley *et al.*, 2005, Zhang and Michniak-Kohn, 2012).

1.1.4 Hypodermis

Despite being recognized by the majority of authors as a subcutaneous tissue, the hypodermis is composed by collagen and elastin fibers. Fibroblasts, adipose cells (as seen in Figure 2) and macrophages can also be founded in hypodermis. Approximately half of the fat stored in the body can be found in this layer, although their amount and location depend of the age, sex and diet. The main functions of the hypodermis are supplying of energy and also assure mechanical strength to skin (Böttcher-Haberzeth *et al.*, 2010, Seeley *et al.*, 2005, Metcalfe and Ferguson, 2007b).

1.1.5 Skin appendages

Skin has a variety of appendages, principally hairs, sebaceous and sweat glands. In most areas of the skin, the sweat glands are simple, coiled tubular glands that secrete a watery fluid onto the skin surface, by the process of exocrine secretion. The coiled, secretory portions of these glands are an important component of the thermoregulatory mechanism in humans. For instance, sebaceous glands secrete sebum to moisturize the skin and hair and even the hair follicles, which are a source of proliferation of keratinocytes during epithelialization, performing an important role in wound healing. So, the sebum acts as a waterproofing and moisturising agent for the hair and skin surface (Lanza *et al.*, 2007, Seeley *et al.*, 2005, Zhang and Michniak-Kohn, 2012, Young, 2006). Moreover, there are nerve fibers that can be both myelinated and unmyelinated. The unmyelinated fibers go from the epidermis up to the granular layer. Myelinated fibers are distributed regularly in the upper dermis with terminations to Meissner corpuscles and Merkel complexes. These nerve fibers are responsible for cutaneous sensations and prevent skin from injury due to heat, cold, pain and pressure. The smooth muscle of the skin, the arrector pili muscle, helps in the erection of the hair

(goose pimples) in cold weather, thereby trapping warm air near the skin, to protect against the cold (Zaidi and Lanigan, 2010).

1.2 Wounds

Wound healing is a highly regulated process of cellular, humoral and molecular mechanisms that starts immediately after an injury (Lazarus *et al.*, 2002, Enoch and Leaper, 2005). According to the US Wound Healing Society, a wound can be described as a result of the "disruption of normal anatomic structure and function" of the skin, resulting from physical or thermal damage or as a consequence of the presence of an underlying medical or physiological condition (Boateng *et al.*, 2008). Wounds can be classified based on the nature of the repair process, on the number of skin layers and area of skin affected. Based on the type of the repair process, wounds can be classified as acute or chronic wounds (Boateng *et al.*, 2008, Li *et al.*, 2007).

Acute wounds are characterized by their ability to heal completely with minimal scarring within the expected time frame, normally 8-12 weeks (Boateng *et al.*, 2008). The newly formed tissue has a similar structure and comparable functions to native skin (Li *et al.*, 2007). Mechanical injuries, due to external factors such as abrasions, caused by frictional contact between the skin and hard surfaces, are the primary causes of acute wounds (Li *et al.*, 2007, Boateng *et al.*, 2008). Other examples of mechanical injuries include penetrating wounds caused by knives, gun shots and surgical procedures. Burns and chemical injuries (caused by radiation, electricity, corrosive chemicals and thermal sources) are another categories of acute wounds (Boateng *et al.*, 2008).

Chronic wounds represent a different kind of challenge for TE (Supp and Boyce, 2005). These are characterized by their slow healing, i.e., that do not heal after 12 weeks of injure occur and often reoccur. Repeated tissue insults or underlying physiological conditions such as diabetes and other malignances, persistent infections, poor primary treatment and other patient related factors, contributes to the disability of these wounds to heal faster (Boateng *et al.*, 2008). These wounds involve a large surface area and have a high incidence in general population, featuring an enormous medical and economic impact (Supp and Boyce, 2005). Pressure and leg ulcers (venous, ischaemic or of traumatic origin) are known as the most common chronic wounds (Boateng *et al.*, 2008, Supp and Boyce, 2005).

Some authors have described wounds both acute and chronic that are difficult to heal as "complex wounds" with unique characteristics, such as: extensive loss of the integument which comprises skin, hair, and associated glands; infection that may result in tissue loss; tissue death or impairment of the signs circulation and presence of pathology (Boateng *et al.*, 2008). There are also anomalies in certain stages of the healing process that can result in

excessive healing (e.g. hypertrophic scars, keloids) (Enoch and Leaper, 2005, Reinke and Sorg, 2012).

Skin functionality must be quickly restored, in order to maintain homeostasis. The scarring of acute wounds involves a complex process comprising a series of dynamic events (Enoch and Leaper, 2005). Healing of acute wounds occurs as a carefully regulated cascade of overlapping processes, that require the coordination of a variety of cellular activities, including phagocytosis, chemotaxis, mitogenesis, and synthesis of components of the ECM (Enoch and Leaper, 2005).

1.2.1 Types of wound healing

In each healing process, there are several mechanisms involved. The severity of the wound, number of skin layers affected and the occurrence or absence of bacterial infection allows us to classify the wound healing in different categories (Gurtner *et al.*, 2008, Enoch and Leaper, 2005, Reinke and Sorg, 2012):

- Primary healing- occurs when a wound is closed within 12-24 hours after its occurrence (e.g. clean surgical incision, clean laceration). The incisions cause only a localized disruption in the basal membrane and the death of some cells of the underlying connective tissue. In this type of healing, epithelial regeneration predominates over fibrosis (Ladin, 2001, Enoch and Leaper, 2005, Gurtner *et al.*, 2008).

- Delayed primary healing- occurs in a contaminated or poorly delineated wound. The skin and subcutaneous tissues are left unopposed and the closured is performed after host defenses have helped to debride the wound. After 3-4 days, the local recruitment of phagocytic cells to the wound occurs and inflammatory cells kill the contaminating bacteria. Collagen metabolism is usually unaffected and the wound retains its tensile strength, as if the wound closure occurred immediately (Enoch and Leaper, 2005, Gurtner *et al.*, 2008, Sun *et al.*, 2011).

- Partial-thickness wounds (superficial healing)- are seen in injuries such as superficial burns and abrasions where the injury involves the epithelium and the superficial part of the dermis. The cells migrate towards each other from the basal layer to surround the wound. The healing occurs purely by epithelialization, and the anatomical and physiological restoration is virtually completed (Enoch and Leaper, 2005, Gurtner *et al.*, 2008, Reinke and Sorg, 2012).

- Full-thickness wounds (secondary healing)- occur in a wound where an extensive loss of soft tissue occurred, as a consequence of a major trauma, severe burns and after some surgical procedures. This type of wounds requires the application of procedures that help the wound contraction and subsequently allows epithelialization. The epithelial cells alone are not able to restore the skin original architecture, so there is ingrowth of granulation tissue from the wound margin, followed by accumulation of ECM with the laying down of collagen (Enoch and Leaper, 2005). Myofibroblasts, which have structural properties similar to that of fibroblasts and a smooth muscle cells, play a crucial role in the healing of this type of wounds (Enoch and Leaper, 2005, Ladin, 2001, Sun *et al.*, 2011, Reinke and Sorg, 2012).

1.2.2 The wound healing mechanism

Wound healing is a complex biological process that includes a wide range of mechanisms such as coagulation, inflammation, matrix synthesis and deposition, angiogenesis, fibroplasia, epithelialization, contraction and remodelling (Reinke and Sorg, 2012, Ladin, 2001, Alemdaroğlu *et al.*, 2006). These events occur in cascade and involve the migration of several cell types at the wound site, during the different stages of the healing process (as seen in Figure 3) (Enoch and Leaper, 2005).

The normal mammalian response to wound injury occurs in four overlapping but distinct phases: haemostasis, inflammation, migration-proliferation and remodelling (maturation) (Reinke and Sorg, 2012, Gurtner *et al.*, 2008).

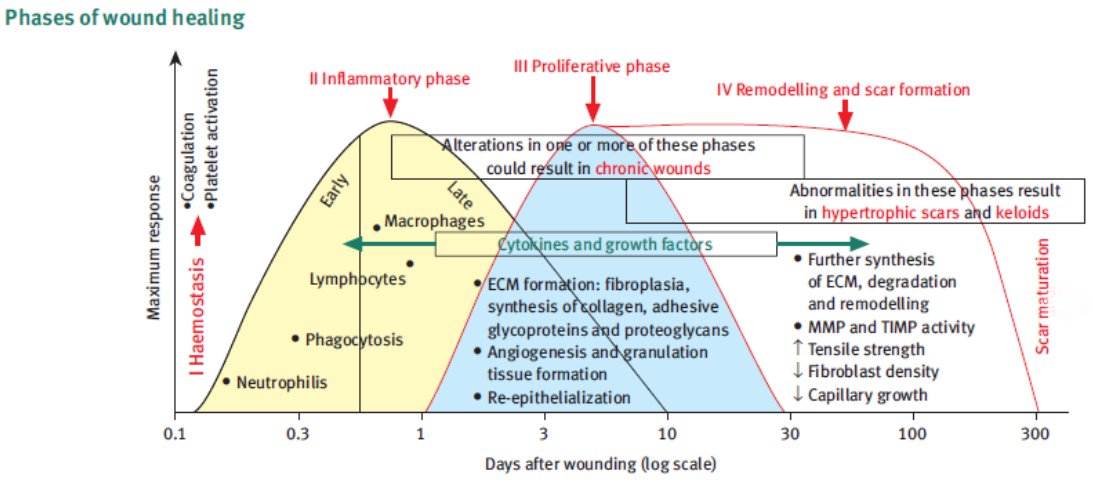


Figure 3 : Phases of wound healing. This mechanism is the result of a complex process, where different types of cells are involved in the different stages of the healing process. Phase I: Haemostasis which is characterized by coagulation and platelet activation; Phase II: Inflammatory phase where the cells of the immune system are recruited to injury site; Phase III: in Proliferation phase occur the formation of ECM, granulation tissue and also angiogenesis; Phase IV: Remodeling, formation and maturation of the scar. ECM: Extracellular matrix; MMP: Metalloproteinases; TIMP: Tissue inhibitors of metalloproteinases (adapted from (Enoch and Leaper, 2005)).

1.2.2.1 Haemostasis

The first stage of wound healing process is dedicated to haemostasis and the formation of a provisional wound matrix. Such event occurs immediately after injury and is completed after some hours (as depicted in Figure 4) (Reinke and Sorg, 2012, Enoch and Leaper, 2005, Gurtner *et al.*, 2008). It begins with the formation of a platelet plug, followed by a fibrin matrix deposition which becomes the scaffold for cell infiltration. There is an invasion of inflammatory cells such as leukocytes, macrophages and neutrophils at the wound site. These cells and platelets release cytokines and growth factors in order to activate the inflammatory process (Reinke and Sorg, 2012).

1.2.2.2 Inflammation

The inflammatory phase of the wound healing cascade occurs immediately after tissue damage. In this stage, components of the coagulation cascade, inflammatory pathways and immune system are all needed for preventing the ongoing blood and fluid losses, to remove dead and devitalized tissues and also avoid infection (Gurtner *et al.*, 2008, Reinke and Sorg, 2012). This phase can roughly be divided into an early phase with neutrophil recruitment and a late phase with the appearance and transformation of monocytes (as shown in Figure 4) (Grose and Werner, 2004, Gurtner *et al.*, 2008). Then, neutrophils are recruited to the wound, and the degranulation of platelets occurs. Moreover, once at the wound site, neutrophils perform their function of killing and phagocytising bacteria and damaged matrix proteins within the wound bed. The role that neutrophils play is crucial within the first days after injury, due to their ability to perform and also secrete proteases, which kills the local bacteria and helps to degrade the necrotic tissue (Reinke and Sorg, 2012). After 2-3 days, monocytes appear at the wound site and differentiate into macrophages. Macrophages get into the injury and support the ongoing process, by performing phagocytosis of pathogens and cell debris, as well as the secretion of growth factors, chemokines and inflammatory cytokines (IL-6, TNF-α) (Gurtner *et al.*, 2008, Reinke and Sorg, 2012, Profyris *et al.*, 2012). Macrophages are involved in host defence, resolution of inflammation, removal of apoptotic cells and support cell proliferation and tissue restoration after injury (Koh and DiPietro, 2011, Reinke and Sorg, 2012).

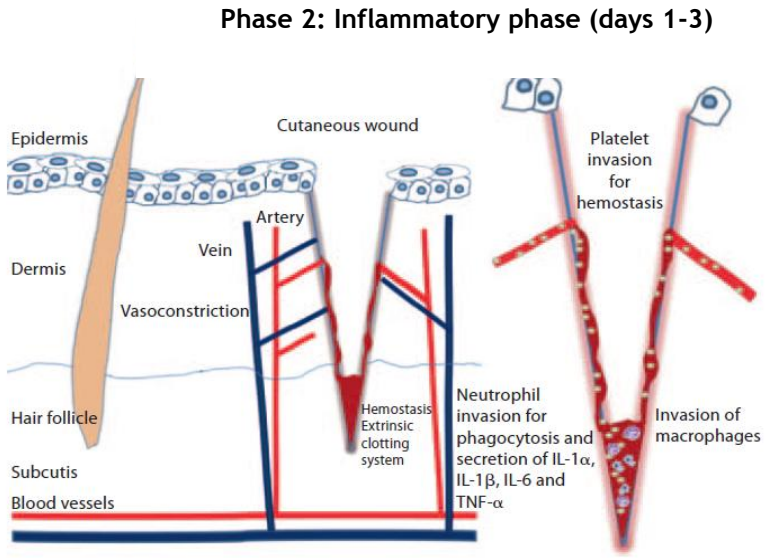


Figure 4: Representation of the inflammatory phase after a skin injury; haemostasis and invasion of inflammatory cells (adapted from (Reinke and Sorg, 2012)).

1.2.2.3 Cell migration and proliferation

Cell migration occurs 2-10 days after injury and is characterized by the migration of different cell types. The migration phase involves the movement of epithelial cells (such as keratinocytes) and fibroblasts to the injured area, in order to replace damaged or lost tissue (Gurtner *et al.*, 2008, Strodtbeck, 2001). New blood vessels are formed, in a process known as angiogenesis (as illustrated in Figure 5) and the sprouts of capillaries associated with fibroblasts and macrophages replace the fibrin matrix by a granulation tissue (Strodtbeck, 2001, Bauer *et al.*, 2005, Reinke and Sorg, 2012).

In the proliferation phase (3-10 days after injury) the main focus of the healing process relies on covering of the wound surface. In this stage, the formation of a granulation tissue occurs and the vascular network is restored (Robson *et al.*, 2001, Strodtbeck, 2001, Bauer *et al.*, 2005, Gurtner *et al.*, 2008). After fibroblasts migrate along the fibrin network, the reepithelialisation starts from the wound edges, neovascularization and angiogenesis processes get activated (Bauer *et al.*, 2005, Strodtbeck, 2001, Reinke and Sorg, 2012, Enoch and Leaper, 2005). Fibroblasts are stimulated by macrophages and some of them are differentiated into myofibroblasts (Opalenik and Davidson, 2005). These are contractile cells that bring the edges of a wound together. Fibroblasts and myofibroblasts interact and produce ECM, mainly collagen type III (Werner *et al.*, 2007). The synthesis of collagen increases throughout the wound, while the proliferation of fibroblasts decreases successively, adjusting a balance between synthesis and degradation of the ECM (Strodtbeck, 2001). The formation of the ECM represents another important step, since it provides support for cell

adhesion, regulates and organizes cell growth, and also allows their displacement and differentiation within it (Reinke and Sorg, 2012, Barker, 2011). The last step of the proliferation phase is the development of the acute granulation tissue, which is characterized by a high density of fibroblasts, granulocytes, macrophages, capillaries and loosely organized collagen bundles (as depicted in Figure 5) (Gurtner *et al.*, 2008, Reinke and Sorg, 2012, Nauta *et al.*, 2011).

At the end of this stage, the fibroblasts assume a phenotype of myofibroblast characterized by large bundles of microfilaments actin disposed along the cytoplasmic face of the plasma membrane of the cells and by linkages cell-cell and cell-matrix. The appearance of myofibroblasts corresponds to the initiation of connective-tissue compaction and the contraction of wound (Hinz, 2007, Singer and Clark, 1999).

The regeneration of any type of tissue requires an interaction between different types of cells, local and systemic mediators such as GFs and hormones, and the ECM where these events occur. GFs are proteins that induce a change in the cellular function by inducing proliferation or differentiation (Werner and Grose, 2003). They promote healing by stimulating the production of components of the basement membrane, preventing dehydration, increasing inflammation and the formation of granulation tissue (Werner and Grose, 2003, Li *et al.*, 2007).

Multiple studies have demonstrated a benefic effect of many of GFs, e.g., platelet-derived growth factors (PDGFs), serine protease thrombin, transforming growth factors (TGF) –α and –ß, and epidermal and endothelial growth factors in the wound healing process. Fibroblasts produce vascular endotelial growth factor (VEGF) and fibroblast growth factor (FGF) (Li *et al.*, 2007, Werner and Grose, 2003).

PDGF was the first growth factor shown to be chemotactic, since it induces cells migrating into the healing skin wound, such as neutrophils, monocytes, and fibroblasts. In addition, it enhances proliferation of fibroblasts and production of extracellular matrix by these cells. Finally, it stimulates fibroblasts to contract collagen matrices and induces the myofibroblast phenotype in these cells (Heldin and Westermark, 1999, Whitney, 2005). PDGF has been suggested to have two major, but distinct, roles in wound repair: an early function to stimulate fibroblast proliferation and a later function to induce the myofibroblasts phenotype (Whitney, 2005).

Furthermore, FGFs are mitogenic for several cell types that are present at the wound site, including fibroblasts and keratinocytes (Werner and Grose, 2003). In addition to their mitogenic effects, FGFs also regulate migration and differentiation of their target cells, and some FGFs have been shown to be cytoprotective and to support cell survival under stress conditions (Ornitz and Itoh, 2001). Numerous *in vivo* effects of FGFs have been demonstrated, which suggest a role of these growth factors in wound repair. In particular, FGF1 and FGF2

were shown to stimulate angiogenesis in various assay systems (Werner and Grose, 2003, Whitney, 2005).

The TGF-β superfamily encompasses a range diverse of proteins, many of which play important roles during development, homeostasis, disease, and repair. Furthermore, TGF-βs are very potent stimulators of the expression of extracellular matrix proteins and integrins. Thus they possess the properties expected of wound cytokines and indeed are among the most studied molecules for wound healing (Whitney, 2005, Li *et al.*, 2007, Werner and Grose, 2003). For example, TGF-β1, TGF-β2, and TGF-β3 strongly promote the migration of fibroblasts and endothelial cells and the deposition of extracellular matrices by fibroblasts, during granulation tissue formation (Li *et al.*, 2007).

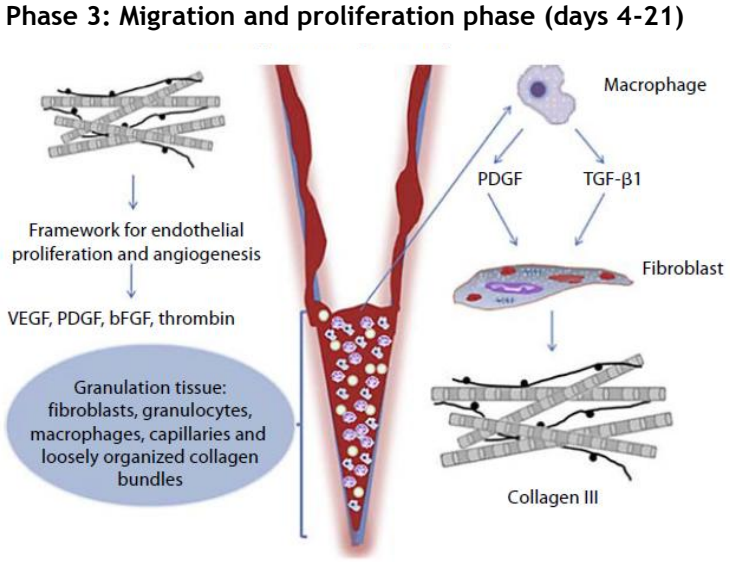


Figure 5: Cell migration and proliferation phase. Fibroblasts and myofibroblasts produce mainly collagen at the beginning of the angiogenesis (adapted from (Reinke and Sorg, 2012)).

1.2.2.4 Remodelling (maturation)

Remodelling is the last phase of the wound healing and occurs from day 21 to up to 1 year after injury. The formation of granulation tissue stops through cells' apoptosis (Enoch and Leaper, 2005, Nauta *et al.*, 2011). During this stage, most of the endothelial cells, macrophages and myofibroblasts undergo apoptosis or exit from the wound, leaving a mass that contains few cells, which is mainly composed by collagen and other ECM proteins (Reinke and Sorg, 2012). During the maturation of the wound, ECM components suffer certain changes (as it is depicted in Figure 6). Collagen type III that was produced in the proliferative phase is now replaced by collagen I. Later on, the myofibroblasts promote wound contraction, through their multiple attachment to collagen and help to decrease the surface of the developing scar (Reinke and Sorg, 2012, Profyris *et al.*, 2012). Finally, the angiogenic

response decreases, the wound blood flow decreases and the acute wound metabolic activity slow down and stop. Subepidermal appendages such as hair follicles or sweat glands are not re-established after serious injury (Reinke and Sorg, 2012, Gurtner *et al.*, 2008, Nauta *et al.*, 2011).

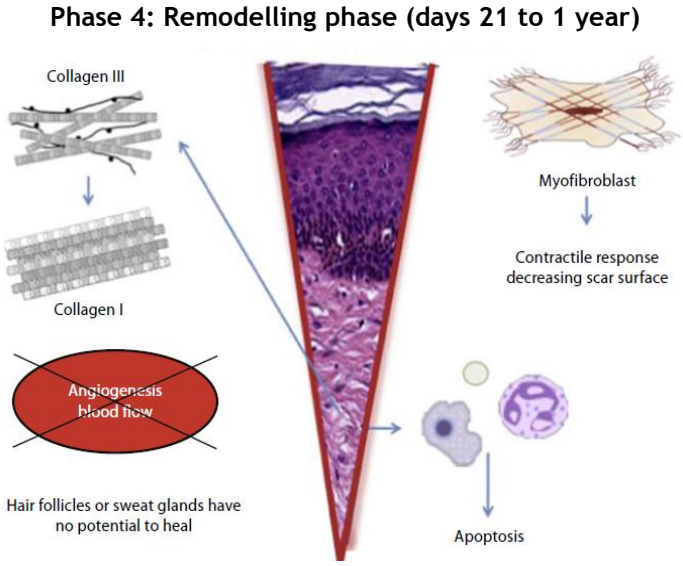


Figure 6: Remodelling phase ; reorganization of the connective tissue and contractile response (adapted from (Reinke and Sorg, 2012)).

1.3 Tissue Engineering

One therapeutic approach of particular relevance to wound healing is tissue engineering (TE), a concept described 20 years ago as "an interdisciplinary field that applies the principles of engineering and life sciences toward the development of artificial tissues. A variety of materials have been investigated to be used as matrices including autologous, allogenic, and xenogenic tissues for tissue engineering purposes (Nair and Laurencin, 2006). To date, the materials used in TE can be natural or synthetic origin: examples of natural materials include alginate and chitosan, collagen, fibronectin, glycosaminoglycans (GAGs), hyaluronan, hydroxyapatites, polypeptides. Such materials have the advantage of be nontoxic and trigger a low chronic inflammatory response. On the other hand, the synthetic materials include polyglycolide, polylactide, polylactide coglycolide and others, that are used for sutures and meshes (Vats *et al.*, 2003, Metcalfe and Ferguson, 2007b). Polytetrafluoroethylene, polyethylene terephthalate and their copolymers are other synthetic materials used for producing new tissues or restore the existing ones (Metcalfe and Ferguson, 2007b). Moreover, materials such as polyurethanes (Fromstein and Woodhouse, 2002, Skarja and Woodhouse, 2000, Guan *et al.*, 2005), poly(diol citrates) (Yang *et al.*, 2004, Yang *et al.*, 2006), polyhydroxyalkanoates (Martin and Williams, 2003, Williams *et al.*, 1999), poly(e-

caprolactone) copolymers (Matsumura *et al.*, 2003, Lee *et al.*, 2003, Jeong *et al.*, 2004), poly(1,3-trimethylnecarbonate)copolymers (Pêgo *et al.*, 2003, Pego *et al.*, 2003) and poly (glycerol sebacate) (Wang *et al.*, 2002) have also been used for developing biodegradable scaffolds with elastic properties in order to produce elastomers that mimic the soft tissue properties. The synthetic nature of materials used in TE has some distinct advantages and disadvantages when compared to natural biological structures.

TE emerged as an essential tool to fill all the problems that exist in current skin substitutes. The application of TE includes three basic elements, i.e., appropriate cells to synthetize the matrices of a new tissue, a three-dimensional (3D) polymeric matrix that provides the suitable environment for the cells to adhere, and growth factors that stimulate cells to regenerate the damage tissue (Lanza *et al.*, 2007, Nerem and Sambanis, 1995, Elias, 2007). Over the last 25 years, TE of the skin has been based on a strong background of material technologies and cell molecular biology (Böttcher-Haberzeth *et al.*, 2010).

The composition and properties of the skin substitute can be much more precisely controlled. Various biomolecules such as GFs and matrix components can be added to skin substitutes for improving their suitability for this particular biomedical application. However, these synthetic skin substitutes generally lack basement membrane and their architecture does not reproduce the native skin structure. The use of non-biological components can be problematic when trying to produce a biologically compatible material (Halim *et al.*, 2010). Moreover they have some limitations, such as lack of cell recognition signals. The synthesis of such materials requires complex procedures, which become expensive for commercial and clinical purposes. Besides, synthetic polymers induce some inflammatory response after implantation (Yang *et al.*, 2006).

One of TE's primary objectives is to recreate an appropriate cellular environment that supports the control and regulation of cells' functions. The best approach is to design a scaffold able to mimic the functions and structure of the naturally existing ECM, in order to promote cell adhesion, proliferation and differentiation to allow the formation of the desired tissue (Lanza *et al.*, 2007, Mohamed and Xing, 2012, Böttcher-Haberzeth *et al.*, 2010). The biomaterials used for the production of these scaffolds must have properties such as:

I. Similar mechanical properties to the injured tissue;
II. Enable or support cell metabolism that build up a new tissue with suitable surface chemistry for cell attachment, proliferation and differentiation;
III. A three dimensional structure with interconnected pores for cellular adhesion, growth, ECM secretion, revascularization and adequate nutrient and oxygen supply;
IV. It must be biodegradable along with the reconstruction of the newly build tissue and the products resultant from its degradation must not affect the tissue regeneration and remodelling process;

V. It must be biocompatible. This property is dependent on the site of implantation, the function and size of the implant and the duration of implantation with a key issue being the time-scale required for material-host tissue interactions to become established.

The scaffold must provide an adequate 3D support for tissue regeneration. While the cells proliferate and differentiate, they produce ECM and scaffolds suffer degradation. Such events can eventually result in the formation of functional tissues (Zhang and Michniak-Kohn, 2012, Böttcher-Haberzeth *et al.*, 2010).

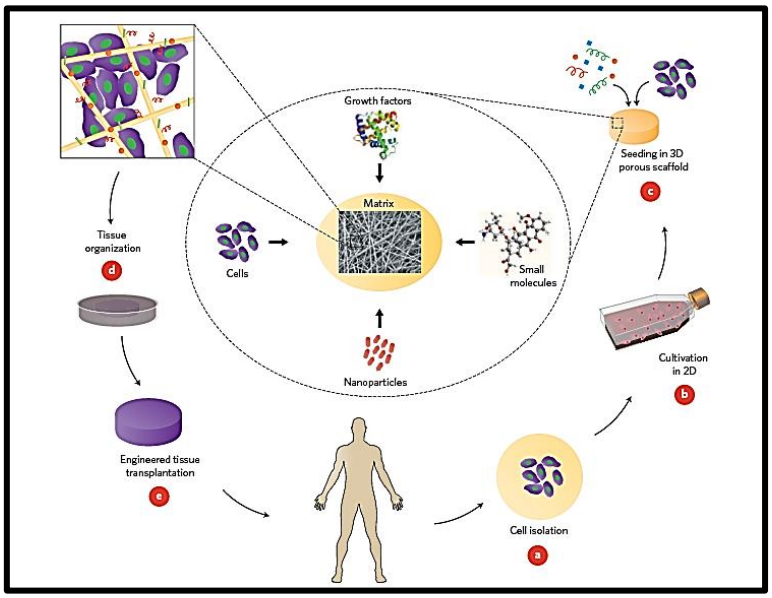


Figure 7: Representation of a tissue engineering concept that involves seeding cells within porous biomaterial scaffolds: a) Cells are isolated from the patient. b) Cell culture *in vitro*. c) Cells are seeded in porous scaffolds together with growth factors, small molecules, and micro- and/or nanoparticles. The scaffolds serve as a mechanical support and a shape-determining material, and their porous nature provides high mass transfer and waste removal. d) Cell constructs are further cultivated in bioreactors to provide optimal conditions for organization into a functional tissue. e) Once a functioning tissue has been successfully engineered, the construct is transplanted into the defect to restore tissue function (adapted from (Dvir *et al.*, 2010)).

The ultimate purpose of tissue-engineered skin grafts is to enable a complete wound regeneration. A 3D supporting framework should serve as a template for tissue regeneration, while simultaneously prevents wound bed contraction, throughout the first stages of healing (Chevallay and Herbage, 2000). These scaffolds mimic the structural environment and serve as systems to simultaneously deliver multiple components, present in the wound microenvironment (e.g. GFs, bioactive molecules and stem cells) in hybrid systems. These 3D structures are generally extracellular-like matrices that may contain cells (fibroblasts and keratinocytes). Another strategy to promote wound regeneration is the exogenous delivery of cytokines (as GFs) and other ligands, for example an arginine–glycine–aspartic (RGD) peptide

matrix has been utilized, underscoring the importance of cell-matrix adhesion in skin repair (Wong and Gurtner, 2012).

Figure 8 shows one potential approach for creating skin constructs in the laboratory. Such technique consists in isolating viable cells from the epidermis and dermis of the human body (keratinocytes and fibroblasts), expand the cell population through *in vitro* culture and then culture these cells on a polymeric matrix (scaffold) that provides a favourable environment for accelerating the wound healing process and prevent the wound from infection. Then, the resulting tissue engineered construct is placed into the animal for *in vivo* assays (Dvir *et al.*, 2010, Lanza *et al.*, 2007). The 3D scaffold should be biodegradable and act like an ECM, since it will temporarily serve as a template for cell adhesion, unhindered proliferation and tissue development. The scaffold should also have an interconnected macroporous network that allows cell penetration and nutrients, oxygen and waste products exchange (Lanza *et al.*, 2007).

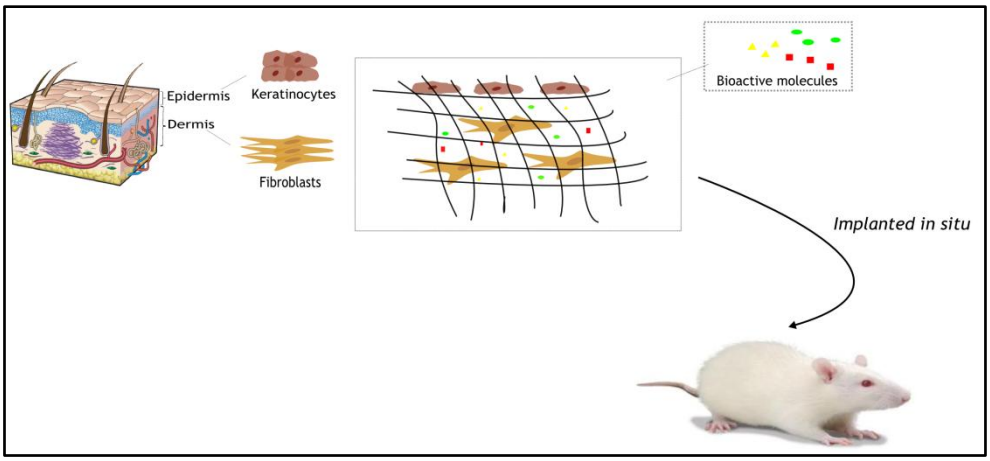


Figure 8: Schematic representation of the production of a skin substitute at the laboratory. After a skin damage occur, the reconstruction of the main layers of the skin (epidermis and dermis) is a key factor for an effective wound healing. Keratinocytes and fibroblasts are fundamental cells in the process of regeneration of the skin. Their incorporation into a polymeric matrix with bioactive molecules will promote the wound healing, in an animal model.

There has been progress in the area of skin TE research and it is anticipated that further novel biomaterials combined with nanotechnology will be applied in this research area. Skin tissue engineering's primary goal is the production of a skin substitute that will support the complete regeneration of a functional tissue with all the appendages and the different layers. Moreover, the reestablishment of the vascular and nervous network in the host at the scar site is other key step in skin regeneration (Mohamed and Xing, 2012).

The ultimate goal of the tissue engineer is to satisfy most, if not all, of these criteria when producing novel, smart skin replacement therapies. Such matrices should attempt to

reproduce the properties of ECM, in order to promote an optimal tissue repair and regeneration of the full thickness wounds (Metcalfe and Ferguson, 2007b, Halim *et al.*, 2010).

1.3.1 Tissue engineering: skin substitutes

Nowadays, the loss or failure of a tissue or organ is the most significant problem in healthcare (Lanza *et al.*, 2007). There are several full-thickness skin injuries resulting from burns, soft tissue trauma and diseases leading to skin necrosis. This represents a significant problem that is far from being solved (Böttcher-Haberzeth *et al.*, 2010). For burns affecting large areas, there is a considerable loss of skin, the development of temporary or permanent skin substitutes appears to be the solution, since the patient's own body is not able to immediately restablish skin arquitecture (Metcalfe and Ferguson, 2007a, Atiyeh and Hayek, 2005).

In the past decades, many skin substitutes such as autografts, allografts and xenografts have been employed for wound healing (Garfein *et al.*, 2003). Autograft skin is harvested from the patient and then placed in the excised areas of the wounded skin of the same individual. Autograft does remain the most frequently used grafting method since it does not induce an adverse immunological reaction from the host (Atiyeh and Hayek, 2005, Babensee *et al.*, 1998). However, there are some disadvantages associated with skin autografts which are the limited availability and the donor site morbidity and scarring (Zhang and Michniak-Kohn, 2012, Babensee *et al.*, 1998). Allograft skin is harvested from an organism of the same species, prior to placement. The use of allograft skin is limited since there is a great risk of disease transmission, eventual immune rejection and difficulties associated with its storage (Babensee *et al.*, 1998). The demand for tissues and organs seriously exceeds the supply, creating a substantial waiting list. Moreover, the immune system tends to reject the foreign tissue or organ (Zhang and Michniak-Kohn, 2012). Xenograft skin is harvested from a different species and the majority of xenograf tissues are excluded due to a vigorous immune response of the patient, that may be caused upon the implantation process, thus leading to a high failure rate (Babensee *et al.*, 1998, Garfein *et al.*, 2003).

Therefore, there is a significant demand of tissues and organs that can be used for regenerative purposes and for being implanted in the human body. In the literature, the skin substitutes have been referred as 3D skin, reconstructed skin, skin equivalents, artificial skin, organotypic culture of skin, or skin grafts (Brohem *et al.*, 2011).

Skin substitutes are a heterogeneous group of wound coverage materials that aid in wound closure and replace the functions of the skin, either temporarily or permanently, depending on the product characteristics (Shores *et al.*, 2007, Halim *et al.*, 2010). There are several important factors that are taken into consideration in the decision to use the skin substitutes in wound management. These include the depth of injury, availability of donor site, presence of wound infection, sites of wound, likelihood of contracture, aesthetic outcome, relative

cost, time consumption and experience of the surgeons (Shakespeare and Shakespeare, 2002). The skin substitutes provide rapid wound dressing that may require less vascularised wound bed, increase the dermal component of healed wound, reduce or remove inhibitory factors of wound healing, decrease the inflammatory response and subsequent scarring (Shores *et al.*, 2007).

In worldwide market of medical and pharmaceutical wound care, the wound dressings primary function was to keep the wound dry by allowing evaporation of its exudates and preventing entry of harmful bacteria. Dressings are classified based on their function in the wound (debridement, antibacterial, occlusive, absorbent, adherence) (Boateng *et al.*, 2008), the type of material employed in its production (e.g. hydrocolloid, alginate, collagen) (Queen *et al.*, 2004) and the physical form of the dressing (ointment, film, foam, gel) (Falabella, 2006). Moreover, dressings can be designed as traditional or modern (moist wound environment) dressings. Traditional dressings include cotton wool, natural or synthetic bandages and gauzes. Unlike the topical pharmaceutical formulations, these are dry and do not provide a moist wound environment (Boateng *et al.*, 2008). Alternatively, the modern dressings are mainly classified accordingly with the materials from which they are produced, including hydrocolloids, alginates and hydrogels, which generally occur in the form of gels, thin films and foam sheets. Their essential characteristic is to retain and create a moist environment around the wound improving the wound healing (Lee and Mooney, 2001, Boateng *et al.*, 2008).

Overall, the biomaterials used in the production of such skin substitutes must be non-toxic, non-immunogenic and can not cause an excessive inflammation. Furthermore, these biomaterials must be biodegradable and be able to support the reconstruction of normal tissue with similar physical and mechanical properties to that of the original skin (Shevchenko *et al.*, 2010).

Skin substitutes functions can be divided in four groups, as previously described by Shakespeare (Shakespeare, 2005):

I. Protection – they can establish a mechanical barrier against microorganisms and prevent water loss;

II. Procrastination - early wound cover is needed until wound closure happens. Several skin grafts or cultured autologous cell applications have been used in extensive burns;

III. Promotion – the delivery of ECM components (collagen, adhesion proteins among others), cytokines and GFs to the wound bed promotes and enhances the natural wound-healing;

IV. Provision - support structures for incorporation of cells and bioactive molecules, which are placed into the wound and stay there during wound healing and/or thereafter (Horch *et al.*, 2007, Shevchenko *et al.*, 2010, Shakespeare, 2005).

There are several skin substitutes that are already available in market (table 1-3) (Shevchenko *et al.*, 2010). The skin-substitute products are classified accordingly to the following parameters:

I. Anatomical structure (dermo-epidermal, epidermal, dermal);
II. Duration (permanent, semi-permanent, temporary);
III. Type of the biomaterial used for their production: biological (autologous, allogenic, xenogenic) or synthetic (biodegradable, non-biodegradable);
IV. Composition regarding the cellular components (cellular, acellular) (Jones *et al.*, 2002, Horch *et al.*, 2007, Atiyeh and Costagliola, 2007, Clark *et al.*, 2007, MacNeil, 2007, Patel and Fisher, 2008).

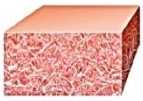
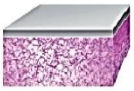
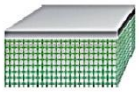
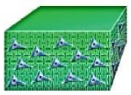
There are skin substitutes that promote only the replacement of the injured epidermis (table 1). The key step for the design and production of an **Epidermal substitute** is to isolate the keratinocytes from a donor and to perform their subsequent *in vitro* culture in order to obtain the necessary number of keratinocytes for therapeutic purposes (Shevchenko *et al.*, 2010). Several epidermal skin substitutes are already commercially available (table 2). For example, MySkin uses subconfluent autologous living keratinocytes which are grown on a silicone support layer with a specially formulated surface coating (Moustafa *et al.*, 2004). On the other hand, CellSpray use either cultured or non-cultured autologous keratinocytes. This technology is based on the possibility of harvesting subconfluent keratinocytes and their subsequent application on the wound bed by a spraying technology. This technology is based on a skin biopsy and a ready-made kit, 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* proliferation and subsequently the wound closure, and cell differentiation to form a epithelial structure without forming a scar (Navarro *et al.*, 2000, Chester *et al.*, 2004).

Table 1: Example of commercially available epidermal skin substitutes. Autologous(auto); Synthetic (synth) (adapted from (Shevchenko *et al.*, 2010)).

	Brand name/manufacturer	Schematic representation	Incorporated human cells	Primary celular loading occurs	Cell source	Scaffold source	Scaffold material	Duration of the cover
Epidermal substitute	Epicel		cultured keratinocyte (confluent cell sheet)	*in vitro*	auto	—	—	permanent
	Myskin		cultured keratinocytes (subconfluent cell sheet)	*in vitro*	auto	synth	silicone support layer with a specially formulated surface coating	permanent
	CellSpray		non-/cultured keratinocytes(subconfluent cell suspension)	*in vitro*	auto	—	—	permanent

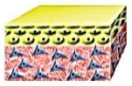
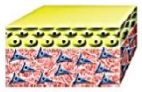
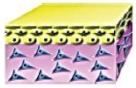
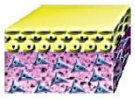
Dermal substitutes are generally acellular, based either on allogeneic, xenogeneic or synthetic materials (Anthony *et al.*, 2006). Table 2 describes some of the dermal substitutes that are already commercially available (Shevchenko *et al.*, 2010). Alloderm is a freeze-dried human acellular dermal matrix, 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 absence of a cellular component (Shakespeare, 2005). SureDerm uses human allogeneic acellular lyophilized dermis (Kim *et al.*, 2003). Integra consists on a porous dermal component made of bovine collagen type I and shark chondroitin-6-sulphate glycosaminoglycan, that are bonded to a silicone pseudo-epidermis (Yannas and Burke, 1980). Dermagraft is composed of polyglactin mesh seeded with living cultured neonatal fibroblasts from foreskin tissue (Shevchenko *et al.*, 2010).

Table 2: Example of commercially available dermal skin substitutes. Autologous (auto); Allogeneic (allo); Xenogeneic (xeno); Synthetic (synth) ; Polyglycolic acid (PGA); Polylactic acid (PLA); Extracellular matrix (ECM); Glycosaminoglycans (GAG) (adapted from (Shevchenko *et al.*, 2010)).

	Brand name/manufacturer	Schematic representation	Incorporated human cells	Primary celular loading occurs	Cell source	Scaffold source	Scaffold material	Duration of the cover
Dermal substitute	AlloDerm		—	*in vivo*	—	allo	human acellular lyophilized dermis	permanent
	SureDerm		—	*in vivo*	—	allo	human acellular lyophilized dermis	permanent
	Integra Dermal Regeneration		—	*in vivo*	—	xeno+synth	Polysiloxane, bovine cross-linked tendon collagen, GAG	semi-permanent
	Biobrane/Biobrane-L		—	*in vivo*	—	xeno+synth	silicon film, nylon fabric, porcine collagen	temporary
	Dermagraft		cultured neonatal fibroblasts	*in vitro*	allo	allo+synth	PGA/PLA, ECM	temporary

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 (Jones *et al.*, 2002). The current commercially available dermo-epidermal (composite) skin substitutes are listed in table 3 (Shevchenko *et al.*, 2010). Karoskin, a human viable cadaveric allograft, is a temporary cover for the wound until it is possible to close it with a permanent skin graft (Shevchenko *et al.*, 2010). 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 of the construct, thus mimicking the normal structure of human skin (Eaglstein and Falanga, 1998, Griffiths *et al.*, 2004). 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 (Shevchenko *et al.*, 2010).

Table 3: Example of commercially available dermo-epidermal skin substitutes. Autologous (auto); Allogeneic (allo); Xenogeneic (xeno) (adapted from (Shevchenko *et al.*, 2010)).

	Brand name/manufacturer	Schematic representation	Incorporated human cells	Primary celular loading occurs	Cell source	Scaffold source	Scaffold material	Duration of the cover
Dermoepidermal substitute (composite)	Allograft (cadaveric)		native	native	allo	allo	native human skin with dermal and epidermal cells	temporary
	Karoskin		native	native	allo	allo	native human cadaver skin with dermal and epidermal cells	temporary
	Apligraf		cultured keratinocytes and fibroblasts	*in vitro*	allo	xeno	bovine collagen	temporary
	OrCel		Cultured keratinocytes and fibroblasts	*in vitro*	allo	xeno	bovine collagen sponge	temporary

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 (Ma *et al.*, 2003, Metcalfe and Ferguson, 2007a, Supp and Boyce, 2005, Ikada, 2006). Their clinical implementation remains riddled with flaws, such as poor preservation and/or restoration of body beauty (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 (Pereira *et al.*, 2007). Bearing this knowledge in mind, there is a huge demand in the development of alternative strategies for the treatment of burns or other types of skin injuries. In the last decade, there has been an increasing interest on the TE approach of dermal and epidermal layers using natural or synthetic matrices (Bhat and Kumar, 2012). For grafting purposes, the ideal skin substitute should promote a faster and better healing (Clark *et al.*, 2007), as well as an enhanced wound closure and a reduced scar formation (Nolte *et al.*, 2008).

1.4 Hydrogels

Hydrogels have become increasingly studied as matrices for tissue engineering due to their hydrophilicity and structural similarity to the ECM (Lee and Mooney, 2001, Sun *et al.*, 2011, Hwang *et al.*, 2010, Zhang and Michniak-Kohn, 2012, Ribeiro *et al.*, 2013). A hydrogel is a network of hydrophilic polymers that swell in water, holding a large amount of it, without losing its structure. Its highly hydrated network provides a biomimetic environment for cellular outgrowth (Hwang *et al.*, 2010, Ribeiro *et al.*, 2013, Cao *et al.*, 2009). Therefore, hydrogels are soft, pliable, wet materials with a wide range of potential for biomedical applications. The high water content of hydrogels renders them biocompatible with the majority of living tissues and their viscoelastic nature, decreases the damage of the surrounding tissue when implanted in the host (Hoffman, 2012). Hydrogels for tissue engineering should provide a 3D microenvironment and therefore contain pores large enough to accommodate living cells. They may also be designed to dissolve or degrade away, releasing GFs and creating pores through which living cells may penetrate and proliferate inside the network. Such pores will also allow the diffusion of gases, nutrients, and waste products. Moreover hydrogels must be biodegradable and biocompatible, i.e., do not induce a chronic immune response (Hoffman, 2012, Cao *et al.*, 2009). While different material dressings have been used to enhance endothelial cell proliferation, the delivery of growth factors through hydrogels may improve the wound healing process (Hunt and La Van, 1989, Ribeiro *et al.*, 2013). Table 4 highlights the important advantages and disadvantages of hydrogels as matrices for TE (Hoffman, 2012).

Table 4: Advantages and disadvantages of hydrogels for being used in TE (adapted from (Hoffman, 2012)).

Advantages	Disadvantages
• Aqueous environment can protect cells and fragile drugs (peptides, proteins, oligonucleotides, DNA) from harmful biologic fluids	• Can be hard to handle
• Good for the transport of nutrient to cells and products from cells	• Usually mechanically weak
• Their surface may be easily modified with cell adhesion ligands	• May be difficult to load drugs and cells and then crosslink them in vitro
• Can be injected in vivo as a liquid that gels at body temperature	• May be difficult to sterilize
• Usually biocompatible	

These hydrogels have been synthesized using natural macromolecules including: agarose, alginate, chitosan, collagen, hyaluronan and fibrin, among others (Lee and Mooney, 2001). These hydrogels can be delivered together with cells into the tissue defects in a minimally invasive manner (Drury and Mooney, 2003). Many studies of hydrogel-based scaffolds are focused on wound healing applications (Balakrishnan *et al.*, 2005, Madsen *et al.*, 2008, Boucard *et al.*, 2007, Ribeiro *et al.*, 2013). Beyond their utility for cell growth, hydrogels can also deliver cytokines, GFs and antibiotics to allow a complete skin regeneration (Qiu and Park, 2001, Shepherd *et al.*, 2011).

When applied to the wound, the use of the hydrogel as dressings does not require a secondary covering because the semi-permeable polymer film controls the transmission of water vapour through the dressing (Boateng *et al.*, 2008). An hydrogel dressing is able to absorb significant amounts of exudate, in order to avoid fluid accumulation, that can lead to skin maceration and bacterial proliferation, that is responsible for a foul smell in infected wounds (Boateng *et al.*, 2008, Drury and Mooney, 2003).

Hydrogels possess most of the desirable characteristics of an "ideal dressing". They are suitable for cleansing of wounds by rehydrating dead tissues and enhancing autolytic debridement. Hydrogel dressings are nonreactive with biological tissue, permeable to metabolites and are non-irritant (Boateng *et al.*, 2008, Kirschner and Anseth, 2013). Hydrogels also promote moist healing, are non-adherent and cool the surface of the wound, which may lead to a marked reduction in pain and therefore have high patient acceptability (Kirschner and Anseth, 2013, Boateng *et al.*, 2008). Some authors have described the ideal properties of hydrogels designed to serve different functions as tissue engineered scaffolds, that might be designed to dissolve or degrade, and also for releasing GFs in the process as well as creating pores into which living cells can penetrate and subsequently, proliferate for replacing the lost or damaged tissue (Hoffman, 2012, Boateng *et al.*, 2008, Kirschner and Anseth, 2013).

While the majority of licensed treatments consist of a synthetic material alone, there is a current trend towards the delivery of cells within the material matrix in order to expedite healing (Hunt and Grover, 2010). Hydrogels can also absorb high quantities of the water without the dissolution of the polymer due to their hydrophilicity, thus giving them physical characteristics similar to that of soft tissues. In addition, hydrogels have high permeability to oxygen, nutrients, and other water-soluble metabolites (Nguyen and West, 2002). The cell encapsulation process offers several advantages, because this strategy is often employable as an injectable system, where cells are suspended in a liquid precursor solution that can be delivered *in vivo* to the site of interest (Nicodemus and Bryant, 2008).

Hydrogels are commonly processed into spherical beads, with sizes ranging from 100 to 2000 μm, while the cells that built the human body are on the order of 100-1000μm, and their components have dimensions in the order of nanometres (Schmidt *et al.*, 2008, Rabanel *et*

al., 2009). Cell-cell interactions and cell-biomaterial interactions occur at the microscale range. Thus, creating devices that will interact with cells at the micro level, will potentially avoid extreme damage of entire tissues or even organs, and also reduce inflammatory response while better targeting and treating the illness (Caldorera-Moore and Peppas, 2009). The spherical nature of the hydrogel beads maximizes the surface area and the small volume of the gel beads facilitate the biomolecular transport (Schmidt *et al.*, 2008).

Cell encapsulation in biopolymer hydrogels was initially investigated for the treatment of diseases through a sustained release of therapeutic molecules. Later this method has been applied in TE (Hunt *et al.*, 2009).

Following the implantation of hydrogels loaded with cells, the immune response can be activated by the adsorption of proteins onto the materials, which will subsequently stimulate the recruitment of immune cells, such as macrophages (Schmidt *et al.*, 2008). These immune cells destroy the transplanted cells through one of the many well documented pathways. The immune response causes structural damage of certain hydrogels due to several intrinsic factors (e.g., enzymes) and extrinsic factors (e.g., external mechanical loading). The immune response also elicits fibrosis around the hydrogels, which subsequently starve any encapsulated cells and limit the efflux of bioactive molecules secreted by the cells (Hunt and Grover, 2010, Nicodemus and Bryant, 2008, Schmidt *et al.*, 2008).

The immune response must be controlled in order to avoid the destruction of hydrogel and encapsulated cells or bioactive molecules and thus impair the wound healing process. To minimize the immune response against the hydrogels, some approaches have been tested (as can be seen in Figure 9) (Schmidt *et al.*, 2008). The immune responses to cell-hydrogel constructs are mediated with the use of biocompatible materials, which present minimal amounts of toxins and prevent protein adsorption of immune cells. Inhibiting protein adsorption onto the hydrogels may hinder the migration of immune cells and subsequently attenuate the threat to the encapsulated cells (Lee and Mooney, 2001, Schmidt *et al.*, 2008). The immune response is also mediated by coating of cell membranes with inert biocompatible polymers. The presence of the layers on the cell membrane does not affect the cell's ability to secrete drug molecules, while providing a thin biochemical and biophysical barrier to the immune system. Combining this cell-membrane coating technique with cell encapsulation techniques, using biocompatible polymers, may further minimize the immune response against cells loaded within hydrogels and extend the lifetime of transplanted cells (Lee and Mooney, 2001, Schmidt *et al.*, 2008).

Encapsulating cells into hydrogels has shown promising results for reducing the immune response and increasing the efficacy and viability of transplanted cells, improving the tissue granulation formation and re-epithelialization (Schmidt *et al.*, 2008).

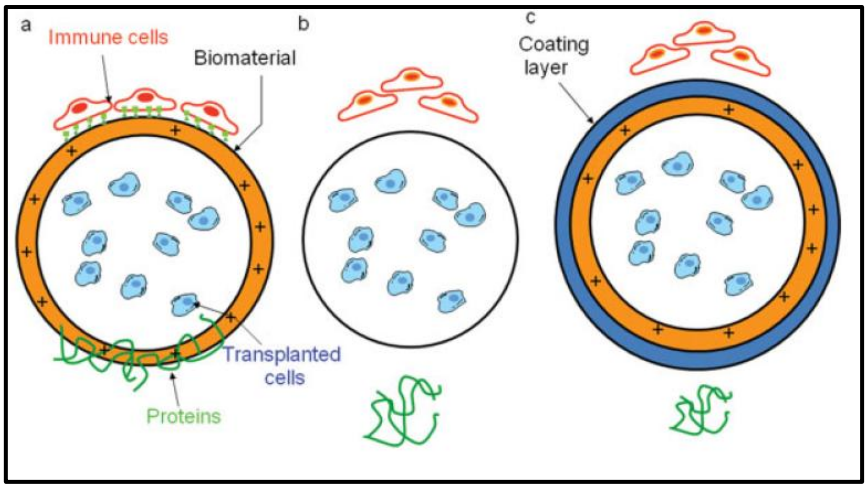


Figure 9: Schematic description of the strategies used to control hydrogel loaded with cells immunogenicity in order to avoid the rejection of hydrogel by patient. (a) Cell encapsulated within biomaterials may allow immune cells and proteins to adhere. (b) The use of biocompatible materials minimizes protein adsorption of immune cells. (c) Coating of biomaterials with some type of polymers avoids the protein adsorption of immune cells (adapted from (Schmidt *et al.*, 2008)).

The demand for organs and tissues for transplantation has increased and TE has searched for alternatives. Numerous strategies are currently been used, where the hydrogels are the main class of materials chosen. Depending on the tissue of interest and the specific application, the required scaffold material and its properties will be quite different (Drury and Mooney, 2003). A variety of synthetic and naturally derived materials may be used to produce hydrogels for TE applications.

Natural polymers have frequently been used in hydrogels production, because they are either components of the ECM or have macromolecular properties similar to it. Natural polymers used in this area of research include agarose, alginate, chitosan, collagen, fibrin, gelatin, and hyaluronic acid (HA) (Lee *et al.*, 2001, Drury and Mooney, 2003). Collagens are the main proteins of the ECM of mammalian tissue and comprise 25% of the total protein mass of most mammals (Lee *et al.*, 2001). Similarly, HA is found in different amounts in all tissues of adult animals (Lodish *et al.*, 2000). Like HA, both alginate and chitosan are hydrophilic linear polysaccharides (Francis Suh and Matthew, 2000). They have also been shown to interact in a favourable manner in vivo and thus have been used as hydrogel scaffold materials for TE (Francis Suh and Matthew, 2000). The gel forming property of alginate helps in removing the dressing without much trauma, and reduces pain experienced by the patient during dressing changes. It provides a moist environment that leads to rapid granulation and reepithelialisation (Paul and Sharma, 2004). Moreover, chitosan is a natural biopolymer that is derived from chitin, a major component of crustacean outer skeletons. This material is known in the wound management field for its haemostatic properties. Furthermore, it also possesses other biological activities and affect macrophage function that helps in enhancing the wound

healing process (Dash *et al.*, 2011). Chitin derivatives possess many unique properties, such as biocompatibility, biodegradability, hydrophilicity, adsorption capability and high reactivity. Moreover, chitin-based polymers are materials with great versatility to be processed in different forms (fibres, sponges, membranes, beads and hydrogels) (Khor and Lim, 2003, Mano *et al.*, 2007). In turn, agarose is a linear polysaccharide constituted by highly flexible molecules that gives them the ability to form different gels at room temperature (Mano *et al.*, 2007).

Synthetic materials include poly(ethylene oxide) (PEO), poly(vinyl alcohol) (PVA), poly(acrylic acid) (PAA), poly(propylene fumarate-co-ethylene glycol) (P(PF-co-EG)), and polypeptides. Synthetic hydrogels are attractive for TE because their chemistry and properties are controllable and reproducible. For example, synthetic polymers can be produced with specific molecular weights, structure, degradable linkages, and crosslinking modes. Such properties determine gel formation dynamics, crosslinking density as well as the material strength and degradation (Drury and Mooney, 2003, Mann *et al.*, 2001, Lum and Elisseeff, 2003).

Nowadays, the interest in hydrogels has been increasing due to the vast variety of polymers that can be used for hydrogel production: (i) polysaccharides, (ii) proteins, and (iii) polyesters. The advance biotechnology field promoted the production of natural polymers by the fermentation of micro-organisms (Widner *et al.*, 2005) or through by enzymatic processes (Kobayashi *et al.*, 2003). However, the largest amount of materials is still extracted from plant, animal or algae sources (Mano *et al.*, 2007).

1.5 Chitosan

As already mentioned, chitosan is currently receiving a great attention for medical and pharmaceutical applications. The main reasons of this growing interest are related to its intrinsic properties. Indeed, chitosan is known for being biocompatible allowing its use in many medical applications such as topical ocular application (Felt *et al.*, 1999), implantation (Patashnk *et al.*, 1997) or injection. Moreover, chitosan is metabolised by certain human enzymes, especially lysozyme. The products resulting from metabolism are not toxic to organism, thus the chitosan is biodegradable (Berger *et al.*, 2004). Due to its positive charges at physiological pH, chitosan is also bioadhesive, which increases retention at the site of application (Berger *et al.*, 2004). Chitosan also promotes wound-healing and has bacteriostatic effects. In addition, chitosan is very abundant, its production cost is low, and is also environmental friendly. In medical and pharmaceutical applications, chitosan is used as a component of hydrogels (Berger *et al.*, 2004, Ueno *et al.*, 2001).

Chitosan was produced for the first time in the 19th century, when Rouget and co-workers reported the process of deacetylation of chitin (Dodane and Vilivalam, 1998). Chitosan is a

linear polysaccharide comprising copolymers of glucosamine and N-acetyl glucosamine linked by (1-4) glycosidic bonds (figure 10) (Pillai *et al.*, 2009, Kumar *et al.*, 2005, Hudson and Jenkins, 2001).

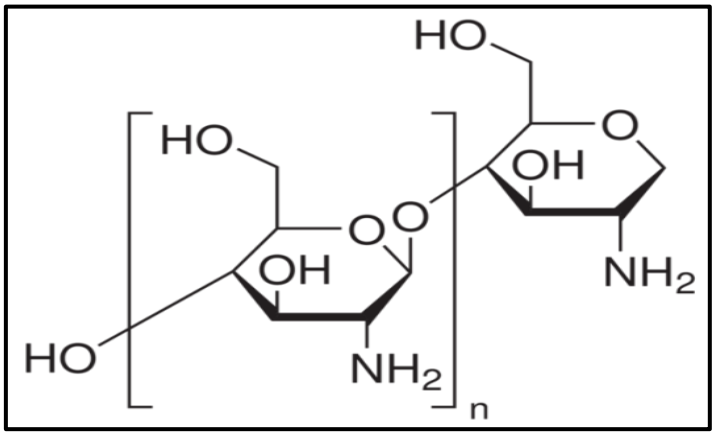


Figure 10: Representation of chitosan structure (adopted from(Hudson and Jenkins, 2001)).

Chitosan has been widely investigated for TE purposes due to its biocompatibility, nontoxicity and biodegradability features (Cao *et al.*, 2009, Ribeiro *et al.*, 2013, Hudson and Jenkins, 2001). *In vitro* studies showed a nonspecific interaction between cells and chitosan. Such characteristics are caused by electrostatic interactions between the positive charges of the amine groups of chitosan and the negative charges of phospholipids of cell membrane (Dillon *et al.*, 2000, Prasitsilp *et al.*, 2000). This electrostatic attraction promotes cells' adhesion, proliferation and differentiation (Francis Suh and Matthew, 2000, Hutmacher *et al.*, 2001). Due to their biocompatibility (Hirano and Noishiki, 2004), biodegradation properties (Muzzarelli, 1997), wound healing promotion (Ueno *et al.*, 2001) and bacteriostatic effects (Felt *et al.*, 2000), chitosan-based materials are promising substitutes for a variety of applications such as drug delivery and cell encapsulation (Ahmadi and de Bruijn, 2007, Di Martino *et al.*, 2005, Kim *et al.*, 2008, Chung and Chen, 2008). Chitosan is available with different degrees of deacetylation (DD), viscosity and molecular weight (Dash *et al.*, 2011). It is readily soluble in dilute acidic solutions (below pH 6.0) (Yi *et al.*, 2005). At low pH, the amines become protonated and positively charged, which makes chitosan a water-soluble cationic polyelectrolyte (Dash *et al.*, 2011), as can be seen in Figure 11.

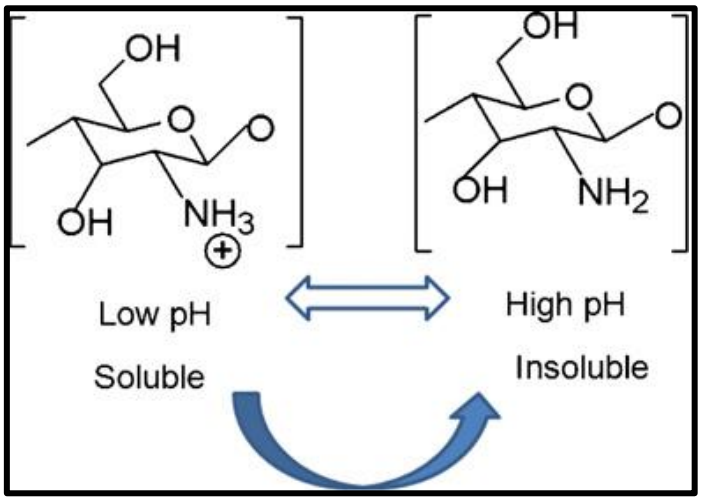


Figure 11: Schematic illustration of chitosan's solubility. At low pH (less than 6), chitosan's amine groups are protonated. At higher pH (above 6.5), chitosan' amines are deprotonated and reactive (adapted from (Dash *et al.*, 2011)).

Chitosan with low degree of deacetylation induces an acute inflammatory response, unlike to the higher DD, which produces a small inflammatory response due to the lower degradation rate. An increase in the DD of the polymer leads to an increase of free amino groups, which allow an effective interaction between chitosan and cells, and consequently stimulates cell's adhesion and proliferation. The deacetylation induces promising biological activities, such as antimicrobial, antitumoral, haemostatic activity and acceleration of wound healing. Moreover, the deacetylation of chitosan enhances their properties such as water solubility, biodegradability, and biocompatibility (Kumar *et al.*, 2005, Ravi Kumar, 2000, Koide, 1998).

Chitosan has been shown to be degraded *in vivo*, which through enzymatic hydrolysis. Lysozyme is the primary enzyme responsible for in vivo degradation of chitosan, which appears to target acetylated residues. The final degradation products are biocompatible chitosan oligosaccharides (Kim *et al.*, 2008).

Another important property of chitosan is its antibacterial activity for different strains, such as: *Enterobacter aerogenes, Salmonellas Typhimurium, Staphylococcus aureus* and *Escherichia coli* (Sudarshan *et al.*, 1992, Wang, 1992, Chen *et al.*, 2002). Due to this antibacterial property chitosan has been blended with other polymers (Kim *et al.*, 2008, Hu *et al.*, 2003). Both chitin and chitosan have been shown to activate the defence system of the host, in order to protect it from pathogens (Sudarshan *et al.*, 1992). The interaction between positively charged chitosan with negatively charged molecules at the cell surface, affects cell permeability (Kim *et al.*, 2008, Chung and Chen, 2008). Furthermore, chitosan can bind to DNA and thereby inhibit mRNA synthesis, via chitosan penetration into the nuclei, thus interfering with the synthesis of mRNA and proteins in microorganisms (Chung *et al.*, 2004, Chung and Chen, 2008, Kim *et al.*, 2008, Dash *et al.*, 2011).

Different studies reported the use of chitosan for the production of skin substitutes. Such approach raises advantages for the wound healing process such as haemostasis, by accelerating tissue regeneration and stimulating fibroblast to synthetize collagen (Taravel and Domard, 1995, Taravel and Domard, 1996, Cho *et al.*, 1999). In the inflammatory phase, chitosan has unique haemostatic properties that are independent of the normal clotting cascades. *In vivo,* this polymer can stimulate the adhesion of fibroblasts, promoting keratinocytes proliferation and modulate the migration behaviour of neutrophils and macrophages, which in turn, modifies the subsequent repairing processes such as fibroplasias and reepithelialisation (Kim *et al.*, 2008, Ribeiro *et al.*, 2009, Dash *et al.*, 2011). Besides, chitosan has also attracted researchers attention due to its excellent mucoadhesive and bioadhesive properties either in hard or soft tissues, that promote the cell-scaffold adhesion and prolonged retention at the site of application, which is essential for regeneration of damaged tissue (Dash *et al.*, 2011).

Chitosan has been used in the preparation of mucoadhesive formulations, improving the dissolution rate of the poorly soluble drugs, drug targeting and enhancement of the peptide absorption (Dash *et al.*, 2011).

In the next generation of skin substitutes, biomaterial scaffolds will be carefully designed to release in a time dependent way, several signalling molecules including growth factors and polypeptide sequences that are involved in cell migration, adhesion, proliferation and differentiation. From this stand point, due to its intrinsic properties it can be stated that chitosan is the most promising candidates to promote a skin regeneration (Kim *et al.*, 2008).

1.6 Hydrogels stimulus-sensitive: agarose

Hydrogels were the first biomaterials designed to be used in the human body. Traditional methods used for hydrogels synthesis include copolymerization and crosslinking of reactive polymer precursors. These methods of hydrogel synthesis only allow a limited control of the hydrogel structure, poor mechanical properties of hydrogel and slow or delayed responses to external stimuli (Kopeček and Yang, 2007, Kopeček, 2007).

Hydrogels can also control drug release by changing the gel structure in response to environmental stimuli. Hydrogels containing such properties can undergo reversible volume phase transitions or gel–sol phase transitions, by changing environmental conditions (Ahmadi and de Bruijn, 2007).

Temperature-sensitive hydrogels, which show phase transitions from sol to gel and gel to sol, according to changes in the temperature, have been studied most extensively for TE applications, as cell-enclosing vehicles, for cell delivery (Gutowska *et al.*, 2001).

Recently, agarose gels have been investigated as delivery vehicles for drugs (Liu and Li, 2005) and living cells for biomedical applications (Yang *et al.*, 1994). Moreover, due to their mechanical properties, similar to that observed in tissues, and biocompatibility agarose gels have been investigated as potential scaffolds for neural (Jain *et al.*, 2006), cartilage regeneration (Gruber *et al.*, 2006) and wound healing (Tripathi *et al.*, 2009).

Agarose is a linear polysaccharide extracted from marine red algae, that can be used to produce hydrogels by increasing its temperature (Buckley *et al.*, 2009). When the temperature of this solution reaches the room temperature, the gelation process occurs. However, such gelation procedure is reversible upon re-heating (Wong and Mooney, 1997). Agarose, as thermoreversible hydrogel, is a linear polysaccharide based on the (1/3)-ß-D-galactopyranose-(1/4)-3,6-anhydro-α-L-galactopyranose unit (Mano *et al.*, 2007). Due to its physical properties, agarose, when soluble in water, forms a gel with a rigid network, resulting on a three-dimensional plastic and porous reticulum. Thus, agarose gel appears like an apyrogenic, colorless and transparent gel, which is viscous- elastic at temperature above 45°C. The specific enzymatic destruction of the polymer is done by α-galactosidase, which is involved in cellular pentose cycle (Cao *et al.*, 2009, Christensen, 2007).

In the form of a hydrogel, agarose has a porous structure (Arnott *et al.*, 1974) and provides a good environment for cellular spreading and proliferation (Martin *et al.*, 2008, Dillon *et al.*, 1998). Furthermore, the pore size and mechanical properties of agarose gels can be easily tailored by varying agarose concentration (Lee and Mooney, 2001). In a previous study, it was shown that an agarose hydrogel matrix enables cell migration (Luo and Shoichet, 2004), which is essential for tissue repair and regeneration (Ridley *et al.*, 2003). The ability of supporting cells' migration through a hydrogel material assures the reestablishment of the normal function of the regenerated tissue (Cao *et al.*, 2009).

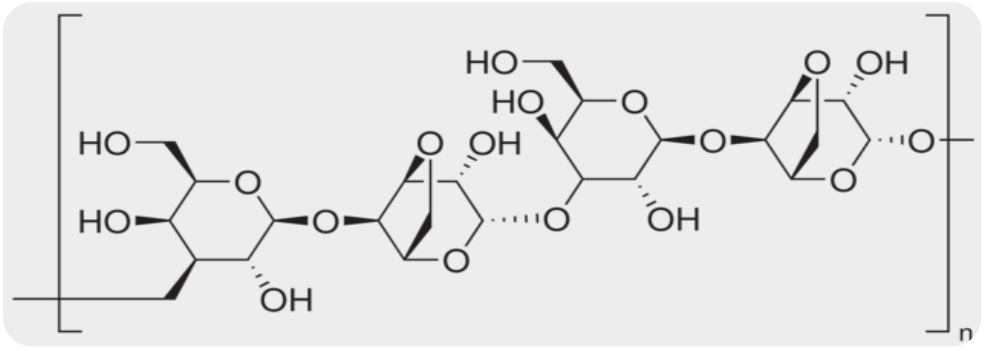


Figure 12: Schematic representation of agarose (adapted from (Mano *et al.*, 2007)).

Furthermore, agarose hydrogel may be polymerizable *in situ*, which is highly attractive for reducing invasiveness of surgery, enhancing the feasibility of *in situ* molding in the required shape improving patient compliance and comfort. Furthermore, it promotes the delivery of cells and signalling molecules to the target site. Moreover, the *in situ* adhesion of the polymer to the surrounding tissue is significantly improved because of intimate contact of the polymer with the tissue (Balakrishnan *et al.*, 2005, Varoni *et al.*, 2012). The ideal injectable implant material should be: (1) highly biocompatible, avoiding any significant inflammatory response; (2) easily injected into the targeted site; (3) able to maintain shape and possess a proper degradation in accordance to tissue regeneration rate; (4) able to carry and maintain cells or other signaling molecules at the implantation site (Eppley and Dadvand, 2006, Varoni *et al.*, 2012).

1.7 Main Goals

In the present study, a new wound dressing (hydrogel) composed by natural polymers, which is responsive to temperature variations, was developed for future application in skin regeneration. The main objectives of this study were:

- Development of chitosan-agarose hydrogel;
- Evaluation and characterization of the physical and biological properties of the produced scaffold through spectroscopic techniques;
- Characterization of the produced chitosan-agarose hydrogel through scan electron microscopy (SEM) analysis;
- Evaluation of the antibacterial activity of the produced hydrogel.

Chapter II

Material and Methods

2. Material and Methods

2.1 Materials

Agarose (low melting point- ultrapure grade) from Nzytech (Lisboa, Portugal). Amphotericin B, Bovine serum albumin (BSA), chitosan (medium molecular weight (MMW)), Dulbecco's modified Eagle's medium (DMEM-F12), ethylenediaminetetraacetic acid (EDTA), LB Broth , Kanamycin, 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 from Pronalab (Barcelona, Spain). Human Fibroblast Cells (Normal Human Dermal Fibroblasts adult, criopreserved cells) were purchased from PromoCell (Labclinics, S.A.; Barcelona, Spain). Clinical *Staphylococcus aureus* isolated. Fetal bovine serum (FBS) was purchased 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 purchased from Fisher Scientific (Portugal).

2.2 Methods

2.2.1 Deacetylation of Chitosan

The chitosan was deacetylated and subsequently, purified to address the influence of the presence of primary amine groups in the chitosan chain in order to improve the surface charges and enhance the interaction with cell (Chatelet *et al.*, 2001, Kumar *et al.*, 2005).
Briefly, the chitosan flakes were dispersed in a NaOH solution. 500mg of medium molecular weight (MMW) chitosan were mixed with 10mL of 1M NaOH. Then the mixture was heated at 50°C, under magnetic stirring, for 4h and then filtered with a 0.44µm filter in a Buchner funnel. The remaining material was washed extensively until the pH was equal to that of ultrapure water. Subsequently, the samples were dried at 40°C overnight (Gaspar *et al.*, 2010).

2.2.2 Production of Chitosan-Agarose hydrogel (CAH)

CAH was prepared following a simple procedure. Hydrogels with a deacetylated chitosan content varying between 0.75% and 2.5%. Briefly, chitosan was first dissolved in 1.0% wt acetic acid solution. Agarose was then added to the chitosan solution, with a final concentration from 1% to 3%, and dissolved by sonication and heating the mixture in a 50°C water bath.

2.2.3 Fourier transform infrared spectroscopy analysis

In infrared spectroscopy the radiation crosses the sample and some of it is absorbed, while other part is transmitted. The resulting spectra represent the frequency of vibration between the atoms linkage from the sample, creating therefore, a specific spectra for those interactions (Bacsik *et al.*, 2004). Fourier-transform infrared spectroscopic (FT-IR) analysis was performed on deacetylated chitosan, agarose and CAH, using a spectrophotometer Nicoletis 20 (64 scans, at a range of 4000 to 1000cm^{-1}) from Thermo Scientific (Waltham, MA, USA) equipped with a Smart iTR auxiliary.

2.2.4 Study of water uptake ability

The swelling properties of CAH were characterized in Tris buffer (pH 5). This hydrogel was placed in an eppendorf with 1 mL of swelling solution and allowed to swell at 37°C. At predetermined intervals, the CAH was weighed. The wet weight of the swollen hydrogels was determined by blotting them with filter paper to remove surface moisture, immediately followed by weighing on an electronic balance, and re-immersed into the swelling medium. The same operation continues until the weights of the swollen microparticles were constant (Zhang *et al.*, 2004). The swelling ratio was evaluated by using Equation 1:

$$Swelling\ ratio\ (\%) = \frac{Wt-Wo}{Wo} * 100 \quad \textbf{(1)}$$

Where Wt is the final weight and Wo is the initial weight of CAH.

2.2.5 Proliferation of fibroblast cells in the presence of CAH

Human dermal fibroblast cells were seed in T-flasks of 25cm^2 with 6mL of DMEM-F12 supplemented with heat-inactivated FBS (10%v/v) and 1% antibiotic/antimycotic solution. After cells attained confluence, they were sub cultivated by 3-5 minutes incubation in 0.18% trypsin (1:250) and 5 mM EDTA. Then cells were centrifuged, ressuspended in a culture medium and then seeded in T-flasks of 75cm^2. Hereafter, cells were kept in culture at 37° C in a 5% CO_2 humidified atmosphere, inside an incubator (Maia *et al.*, 2009, Ribeiro *et al.*, 2009). To evaluate cell behaviour in the presence of the hydrogel herein produced, each formulation of CAH was added (n=5) into a 96-well cell culture plates, in amounts of 25 µL. 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 2h. Human dermal fibroblasts cells were seeded into 96-well plates, containing the hydrogel, at a density of 2x10^4 cells/cm^2 per well, for 24 and 72 hours. Cell growth was monitored using an Olympus CX41 inverted light microscope (Tokyo, Japan) equipped with an Olympus SP-500 UZ digital camera.

2.2.6 Characterization of the cytotoxic profile of the hydrogel produced- MTS assay

In order to evaluate the cytotoxicity of hydrogel, a MTS assay was performed. CAH herein produced were applied into a 96 well plate (n=5) and irradiated under UV light for 30 minutes before cell seeding. Human dermal fibroblast cells were seeded in a 96 well plate containing the biomaterials, at a density of $2x10^4$ cells per well (Ribeiro *et al.*, 2009). The plate was incubated at 37ºC, under a 5% CO_2 humidified atmosphere. After an incubation of 24 and 72h, the medium of each well was then removed and replaced with a mixture of 100µL of fresh culture medium and 20µL of MTS/PMS (phenazine methosulfate) reagent solution. The cells were incubated for 4h, at 37°C, under a 5% CO_2 humidified atmosphere. Cell viability was assessed through the reduction of the MTS into a water-soluble brown formazan product. The absorbance was measured at 492nm using a microplate reader (Sanofi, Diagnostics Pauster). The values of absorbances obtained for formazan are directly proportional to the metabolic activity of cells, which is also directly proportional to the number of viable cells. Wells containing cells in the culture medium without materials were used as negative control. EtOH 96% was added to wells containing cells as a positive control (Ribeiro *et al.*, 2009, Palmeira-de-Oliveira *et al.*, 2010).

2.2.7 Evaluation of antimicrobial activity of CAH

The antimicrobial activity of the CAH was assessed through a standart microdilution method, in order to determine the Minimum Inhibitory Concentration (MIC), which is defined as the lowest concentration of material that inhibits the growth of an organism (Andrews, 2001). To evaluate the antimicrobial effect of the CAH, *Staphyloccocus aureus* was used as a model of Gram-positive bacteria. The culture medium (LB Broth) was inoculated with *Staphyloccocus aureus*, at a concentration of $1x10^6$ colony-forming units (CFU)/ml, was added to several amounts of CAH (25-100µl) with concentrations of chitosan between 25–750 $\mu g/mm^3$. A negative control was prepared without CAH and a positive control was prepared with addition of the Kanamycin antibiotic (2µL). The plate was incubated 24h at 37°C. To monitorize bacterial activity, 10 µL of resazurin (0,1%) was added, after 2-4 hours the fluorescence was measured with a fluorescence plate reader with filter set Ex545/Em590. All experiments were performed with an n=5. The *Staphylococcus aureus* proliferation at CAH surface was also characterized through the Scanning electron microscopy analysis (SEM) analysis in order to evaluate the bacterial growth at surface of the hydrogel. The tested samples with defined concentrations were placed on the surface of a plate of LB agar, in contact with *Staphylococcus aureus* at a concentration of $1x10^8$ CFU/ml, without antimicrobial agents. Then the petri plate was incubated for 24h at 37°C. After that the morphologies of CAH with/without *Staphylococcus aureus* were prepared, as described in section 2.2.8.

2.2.8 Scanning electron microscopy analysis

The morphologies of CAH with/without adhered fibroblasts cells were characterized by SEM. All samples were fixed overnight with 2.5% glutaraldehyde in PBS, at 4°C, and then the samples were dehydrated in graded ethanol of 70, 80, 90, and 100%, 10 minutes each. Finally the hydrogels were frozen in a glass container using liquid nitrogen and freeze-dried for 3 h and subsequently mounted on an aluminium board using a 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 (Ribeiro *et al.*, 2009).

2.2.9 Confocal microscopy analysis

To evaluate the viability cell in the presence of CAH, a confocal scanning laser microscopy (CLSM) analysis was performed. Previously, human dermal fibroblast cells were seeded in a special plate with glass bottom with CAH, at a density of $10x10^3$ cells/ml. After 24h, the samples were incubated for 15 min at 37ºC, immersed in PBS, where 1µl of Propidium Iodide (PI) (1mg/ml), which appears red fluorescent, were added. After incubation, the PI solution was removed and the hydrogel was washed three times with PBS and finally visualized in a Zeiss LSM 710 confocal microscope. Confocal images were obtained, with a Zeiss LSM 710 laser scanning confocal microscope (Carl Zeiss., USA) equipped with a plane-apocromat 10x, 40x and 63×/DIC objectives. The cell nuclei was stained with a red fluorescence and the hydrogel appeared with a blue fluorescent, once it presents self-fluorescence at 461nm. To obtain enough data for 3D reconstruction, a series of sequential slices with different slices thickness (µm), were acquired along the Z-axis, using optimized pinhole parameters in order to comply with the Nyquist-Shannon sampling theorem and minimize image aliasing during acquisition. All the acquired Z-stacks were open as a merged file in the LSM 710 software (Carl Zeiss SMT Inc., USA) where subsequent 3D reconstruction was performed (Gaspar *et al.*, 2011).

2.2.10 Statistical analysis of the results

Statistical analysis of the results MTS assay and MIC was performed using one-way analysis of variance (ANOVA) with the Dunnet's post hoc test and Newman- Keuls multiple comparison test. Each result is the mean ± standard error of the mean of at least three independent experiments.

Chapter III

Results and Discussion

3. Results and Discussion

3.1 Characterization of the morphology of CAH

During this MSc work plan a new termoresponsive hydrogel was developed and optimized for being used in wound healing treatment. To achieve this goal, hydrogel was produced with two natural polymers, namely chitosan and agarose.

Hydrogels are an attractive scaffolding material because their mechanical properties can be tailored to mimic those natural tissues. In general, the crosslinked structure of hydrogels is characterized by junctions or tie points, which may be formed from strong chemical linkages (such as covalent and ionic bonds), permanent or temporary physical entanglements, microcrystalline formation, and weak interactions (such as hydrogen bonds) (Slaughter *et al.*, 2009).

Macroscopic images of the produced thermoresponsive hydrogel are shown in Figure 13. The CAH was obtained by mixture of chitosan and agarose at 40-50ºC (Figure 13A). The gelification was obtained when temperature decrease (Figure 13B). The aspect of the solution changes from transparent to opaque when temperature decreased, which was expected, once the agarose gels are in opaque white color at room temperature. Another change observed was the increased of stiffness due to linkage between water molecules, as described by Kara and collaborators (Kara *et al.*, 2011).

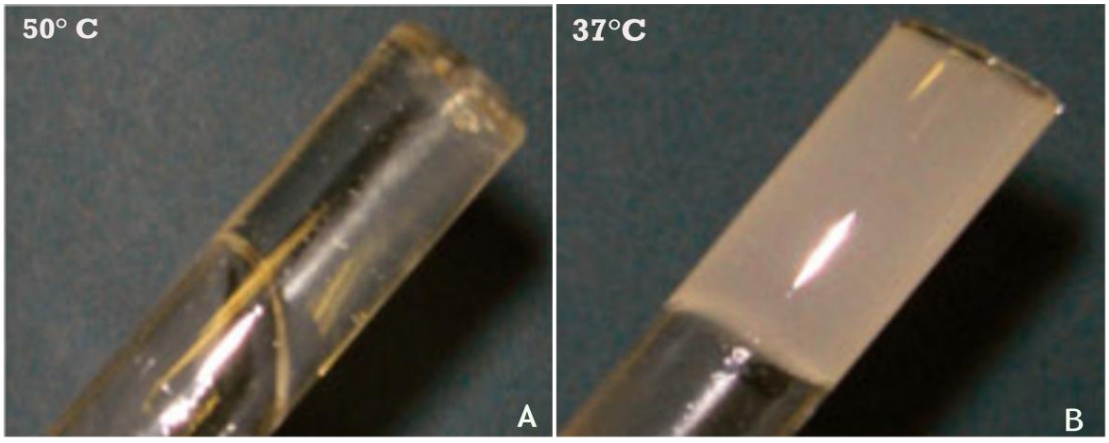


Figure 13: Macroscopic images of CAH at 50°C (A) and 37°C (B).

The morphology and structure of CAH was also characterized by SEM and CLSM. In SEM, (Figure 14), it can be observed that the produced hydrogel exhibits the irregular surface that allows the adhesion and proliferation of cells. Their inner structure is constituted by pores large enough to enable the cell accommodation inside the hydrogel and the diffusion of the nutrients and water, as represented in Figure 14F.

In Figure 14C, the concentration of chitosan is lower than that of the hydrogel presented in Figure 14F, so the size of the pores increase with the concentration of chitosan, which has also been verified by others researchers (Hsieh *et al.*, 2007). During the formation of the hydrogel, when the concentration of chitosan is increased, there are more amino groups in the surface available for interaction with agarose, and the crosslink occurs, while temperature decreases, in order to obtain a porous structure.

It is also possible, through confocal microscopy, to visualize the inner structure of CAH and confirm the presence of macropores (Figure 15), as previously observed in SEM analysis. These hydrogels facilitate the unhindered diffusion of solutes and nutrients owing to the presence of interconnected macropores. CAH, represented in Figure 14F and 15, possesses continuous interconnected pores up to 400-500 μm. These results are in agreement with experimental data previously obtained by other researchers (Ma *et al.*, 2003).

The interconnectivity can provide more space and increased surface area-to-volume ratio of hydrogel scaffolds for cell growth, tissue invasion, local angiogenesis, and facilitate nutrient transport (Huang *et al.*, 2011, Ribeiro *et al.*, 2013). Keeping in mind, the wound dressing application, the interconnected section of CAH promotes drainage, prevents the build-up of exudates, and may be an optimum wound bed for autografting (Ribeiro *et al.*, 2009).

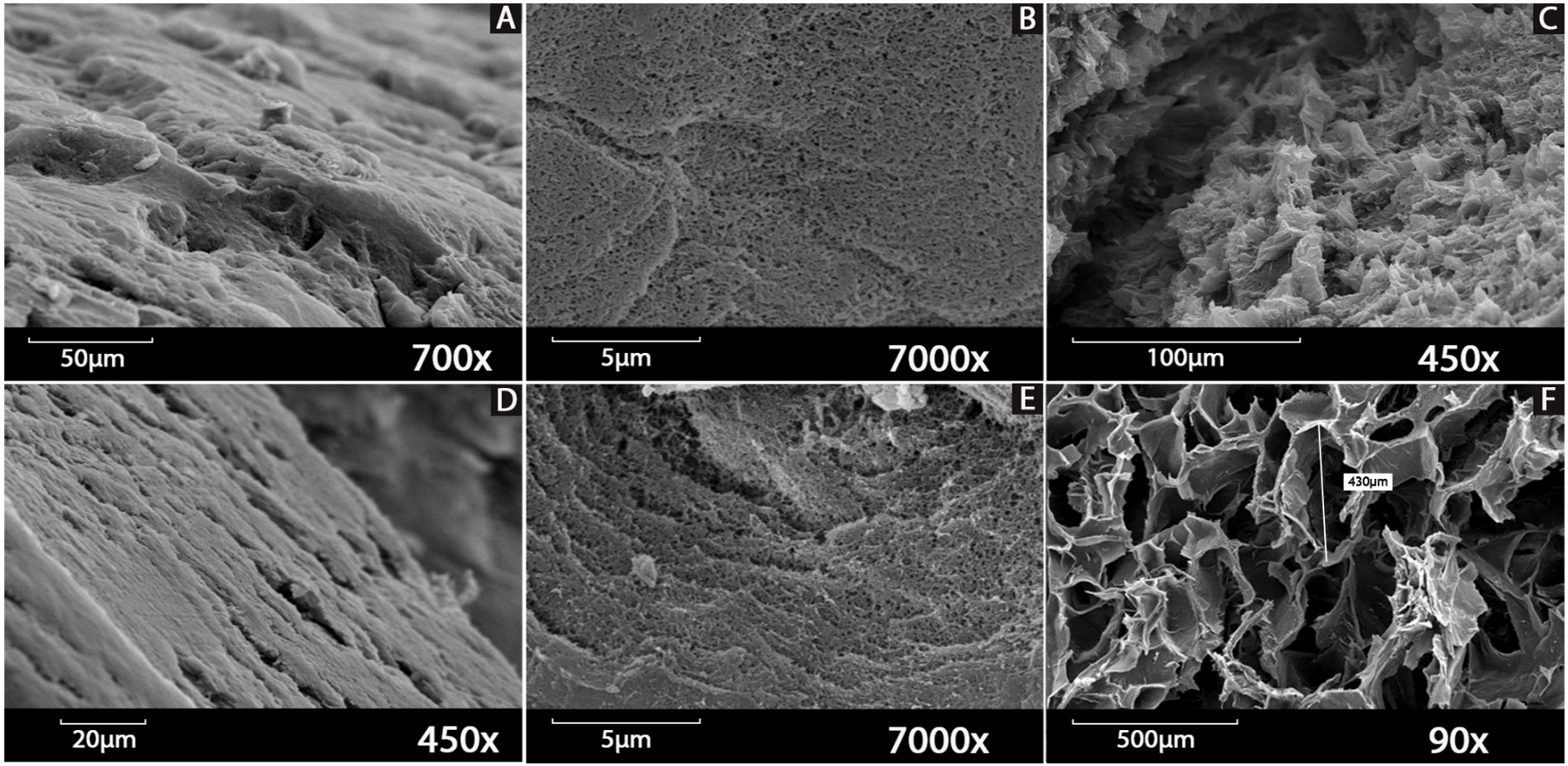


Figure 14: SEM images of CAH: cross section of the CAH (0.75%Ch) 700x (A); surface of CAH (0.75%Ch) 7000x (B); inner structure of CAH (0.75%Ch) 450x (C); cross section of the CAH (1.5%Ch) 450x (D); surface of CAH (1.5%Ch) 7000x (E); inner porous network of CAH (1.5%Ch) 90x(F).

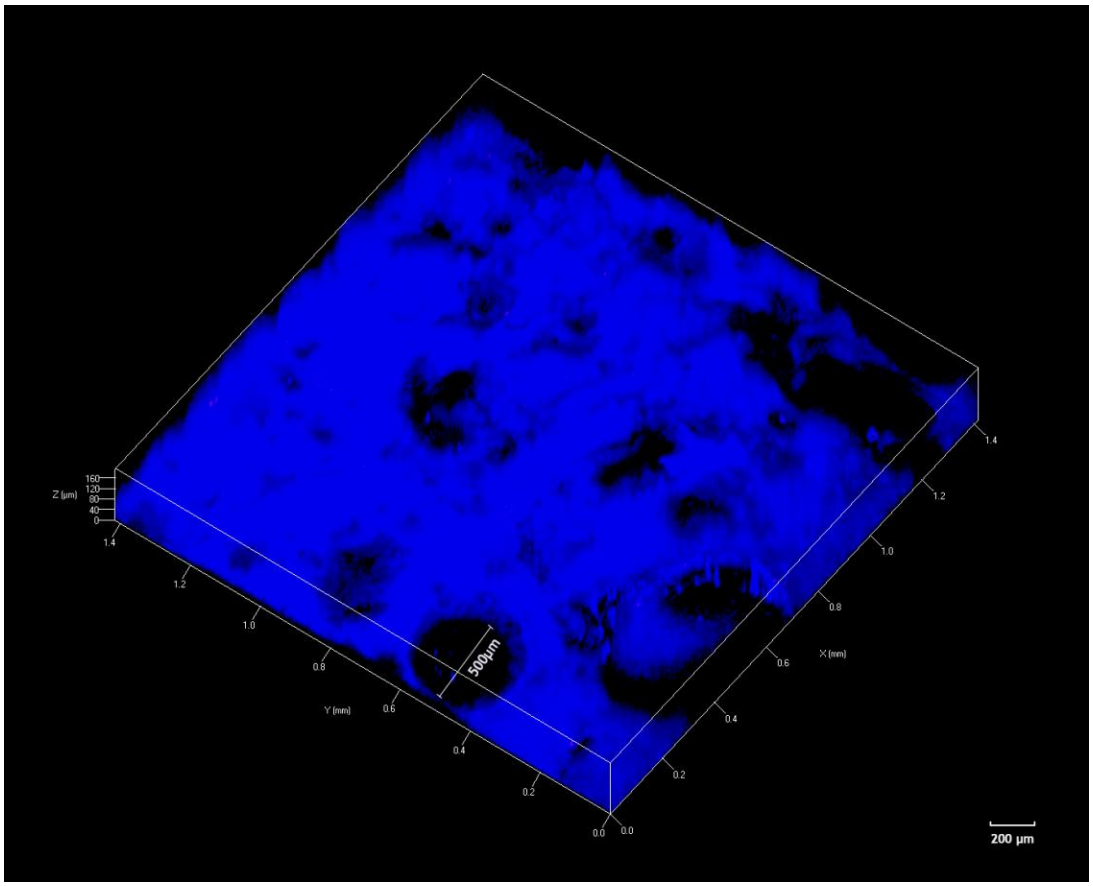


Figure 15: CLSM images of chitosan-agarose hydrogel (1,5%Ch). Scale bar: 200µm.

3.2 FTIR-analysis of CAH

FTIR analysis was performed in order to evaluate the interaction between chitosan and agarose in the produced hydrogel. The FTIR spectrum of the commercial chitosan, presents two absorption peaks at 1650 and 1592 cm^{-1}, which belong to the C-O stretch of the secondary amide and the N-H bending of the primary amine, respectively, as Lim and Hudson previously described (Lim and Hudson, 2004). The other characteristic peak is at 3350 cm^{-1}, that was confirmed by the presence of chitosan owing to the NH group stretching in the polysaccharide, as referred by Bhat and collaborators (Bhat *et al.*, 2011).

The spectrum of the deacetylated chitosan is similar to the spectrum of the commercial chitosan, although there is a variation in the wavelength of the peak from 1592 to 1586cm^{-1}, indicating that the secondary amide (—NH—R) has been further changed to primary amide (—NH_2) by alkaline deacetylation, as is depicted in Figure 16C (Lim and Hudson, 2004). The process of deacetylation of chitosan was achieved, thus it is possible ensure an increase of amine groups at its surface. The electrostatic interaction between the positive charges of the amine groups of chitosan and negative charges of phospholipids of cell membrane is more efficient. By this way the properties of the chitosan are enhanced.

The characteristic peaks of agarose (Figure 16B) were observed at 3359 cm^{-1} (-OH stretching of the hydroxyl group), 1042cm^{-1} (C-O) stretch was attributed to the deformation mode of the C–O groups in the sugar molecules, 1636 (N-H) and 929 cm^{-1} (vibration of C-O-C bridge of 3,6-anydro-L-galactopyranose), similar to results presented in others works (Teng *et al.*, 2009, Bhat *et al.*, 2011). In the produced CAH (Figure 16A), there are two evident peaks at 3347-3332cm^{-1} and 1636cm^{-1}. The first represents the H-bonded of the NH stretch of chitosan and the OH stretch of agarose, as described by others researchers (Teng *et al.*, 2009). The –OH and –NH_2 groups in the chitosan were expected to form hydrogen bonds with the –OH groups of agarose. Moreover, the reciprocal entanglement between the macromolecular chains may create a chitosan–agarose complex (Teng *et al.*, 2009). The second peak shows the NH_2 stretch of the primary amide groups of chitosan (Bhat *et al.*, 2011).

One important detail is that the characteristic absorption bands of the C–O groups do not appear in the CAH and a simple accumulation of the bands in chitosan and agarose was not noticed, therefore suggesting a strong interaction and good compatibility between the two components of the hydrogel (Teng *et al.*, 2009, Bhat *et al.*, 2011).

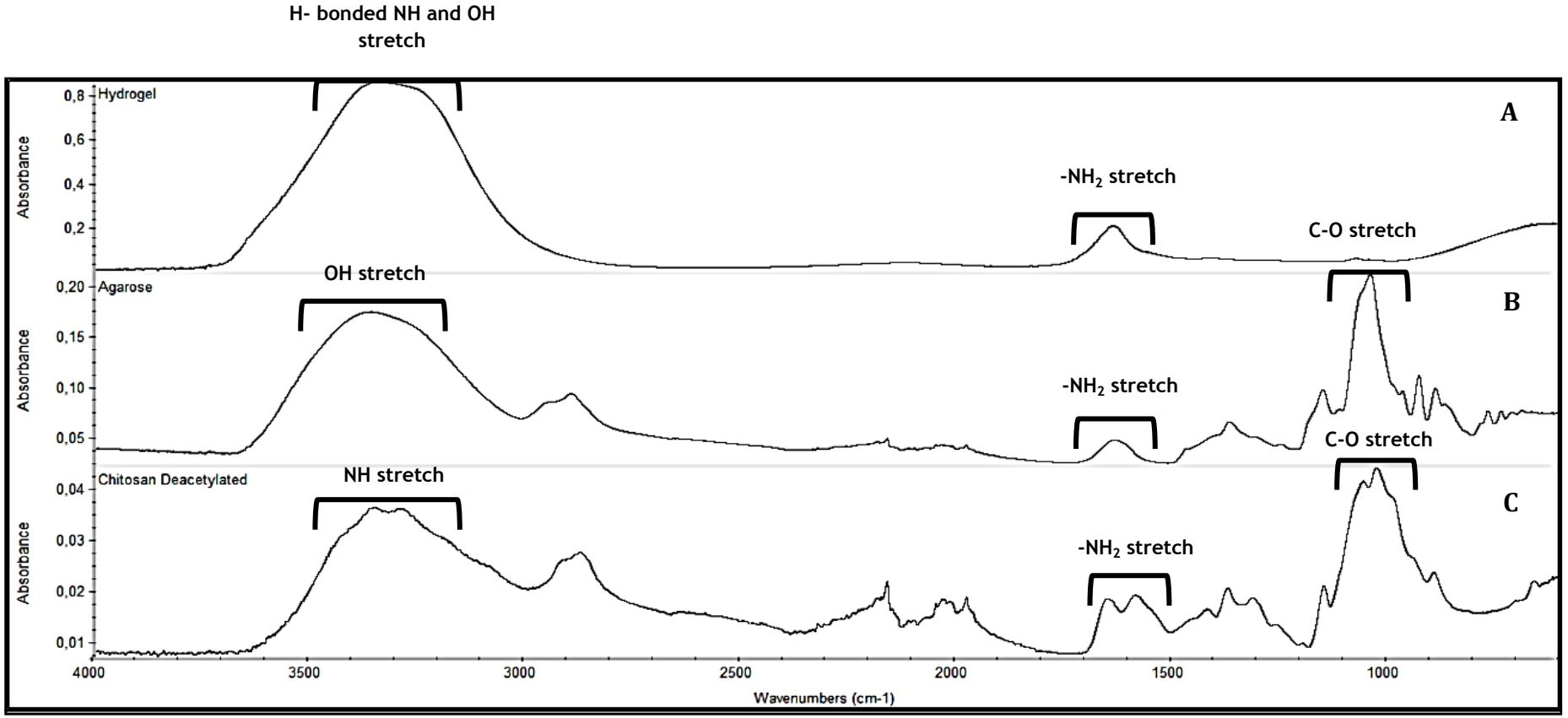


Figure 16: FTIR spectra of the produced CAH and the compounds used for their production: C-A hydrogel (A); agarose (B); Deacetylated chitosan (C).

3.3 Swelling behavior of CAH

The biocompatibility of hydrogel is generally attributed to their hydrophilic character. The diffusion of water into hydrogel matrices is faster than degradation, therefore the matrices begin to swell prior to degradation.

This swelling capability is also responsible for the polymeric matrix expansion, which leads to an increase of the pore size and to the subsequent variation of the scaffold's morphology. Whatever the type of structure, networks containing covalently cross-linked chitosan are considered porous (Capitani *et al.*, 2001, Berger *et al.*, 2004). This term is used to describe networks containing free water that can diffuse through the hydrogel.

The porous covalently cross-linked hydrogels can be used as drug delivery systems from which drugs are released by diffusion (Berger *et al.*, 2004). Indeed, the swelling capacity influences the mesh size of the network. The swelling ratio change of chitosan results in a change in the mesh size of the gel, which modulates drug, bioactive molecules or cells release (Valente *et al.*, 2012, Berger *et al.*, 2004).

The swelling data of the CAH produced herein is shown in Figure 17. It shows that the swelling ratio is positive for CAH, which is indicative of their hydrophilic character. The CAH presented a higher swelling capacity (200%) after 12 hours, which results in increase of the polymeric mesh, allowing the diffusion of nutrients, cells, bioactive molecules and waste, which is essential in skin regeneration. This hydrogel tends to absorb water (free or bulk water) in order to fill the void regions of the polymer network, within the beads that remained dehydrated, until they reach the equilibrium state. This hydrogel behaviour has already been described by other researchers, which reported that the relaxation of the polymeric network is caused by the osmotic pressure (Pasparakis and Bouropoulos, 2006, Valente *et al.*, 2012, Berger *et al.*, 2004). After that, the capacity to retain water decreases to values around 100%, i.e. the hydrogel did not disintegrate during 230 hours. Taking this into account, the swelling studies are very important to understand tissue behaviour when scaffolds are applied for tissue regeneration purposes.

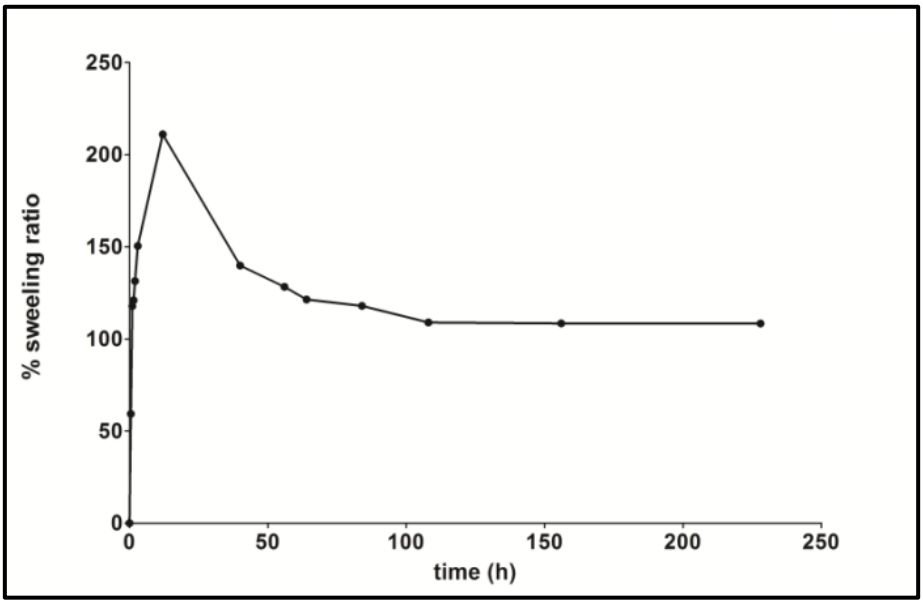


Figure 17: Swelling profile of the produced chitosan- agarose hydrogel.

3.4 Evaluation of viability and cell proliferation in contact with CAH

The viability of CAH was characterized through *in vitro* studies, in order to evaluate the applicability of CAH for the envisioned wound dressing application. As already mentioned, the human fibroblast cells were seeded at the same initial density in the 96-well plates, with or without hydrogel, to assess the CAH cytoxicity. Fibroblasts cells were chosen, due to their potential for skin regeneration, since they synthetized proteins (e.g. collagen and fibronectin) of the ECM, cytokines and GFs that are essential for the wound healing process.

After 24h and 72 hours, cell adhesion and proliferation was visualized using an inverted light microscope. Cell adhesion and proliferation was noticed in wells, where cells were in contact with different CAH and in the negative control (K^-), after 24 hours and 72 hours (Figure 18). In positive control (K^+), no cell adhesion or proliferation was observed. Dead cells with their typical spherical shape are shown in Figure 18.

SEM images were also acquired to further examine and characterize the cell adhesion and proliferation at the surface of CAH. Filopoidia was observed, which is confirmed by presence of cytoplasmic projections in migrating cells. They contain actin filaments that are involved in adhesion between cell and surface of the hydrogel. As expected, a higher concentration of chitosan promotes better cell adhesion, such as can be observed in SEM images, and as described in literature (Koide, 1998, Ravi Kumar, 2000). After 72h, cells grew and remained viable at the surface of CAH (Figure 19).

To further characterize the possible accommodation of fibroblast human cells inside of inner structure of CAH, confocal microscopic images were also acquired. CLSM is an optical microscopy technique that has found tremendous utility in biology, biophysics, chemistry and materials science (Gough, 2008). This technique offers many advantages over the conventional optical microscopy, including enhanced contrast and 3D analysis. Imaging with a CLSM provides a means to collect high-resolution optical images without the incorporation of out of focus light or scattered light (Gough, 2008). The principle behind CLSM is to scan a focused laser beam through a sample and collect the reflected or emitted light from the sample, while removing any light originated from the outside of the focal point of the laser beam. The CLSM can collect images of individual slices using fluorescence or reflection from a sample in the xy, xz and yz planes (Gough, 2008).

Human fibroblasts cells were seeded in contact with CAH, after 24h, cells were labelled with the red molecule fluorescent PI, for 15 min. Then, to evaluate cell growth within the porous structure of CAH, CLSM images were obtained. Through the analysis of CLSM images obtained (figure 20A and 21A), it can be observed cells within CAH. These images also give an idea of cells location inside of the CAH, and the porous was sufficiently large for accommodate human fibroblast cells.

In figure 20B, it is possible to observe cells in scaffold's structure. Figure 21B presents the orthogonal projection, where cell internalization in CAH is more perceptible. However, it was also noticed that there are more cells in CAH periphery. Such feature can be explained by the greater diffusion of both oxygen and nutrients that are essential for cell survival, which occurs better at the edges of the hydrogel. The results obtained showed that structure of CAH was effective for internalization of human fibroblasts cells.

Based on the obtained results we can conclude that CAH is biocompatible and promote cell proliferation along time. Hydrogel surface characteristics supports cell adhesion and also allow cells internalization in their structure. Human fibroblast cells were able to accommodate within polymer matrix of the hydrogel. Beside the biological factors, the microstructure of the scaffold plays an important role in diffusion of nutrients, cell proliferation ability and tissue vascularization. All these features are essential for improving and accelerate of the wound healing process (Bhat *et al.*, 2011, Bhat and Kumar, 2012).

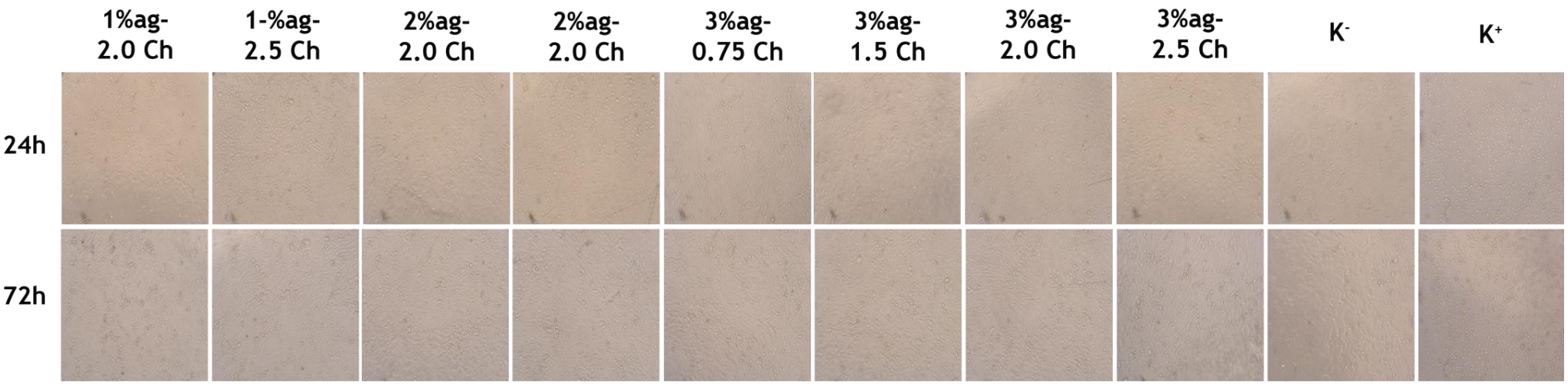


Figure 18: Microscopic images of human fibroblast cells after being seeded in the presence of the CAH during 24h and 72h; agarose (ag); Chitosan (Ch); negative control (K) (live cells); positive control (K^+) (dead cells). Original magnification 100x.

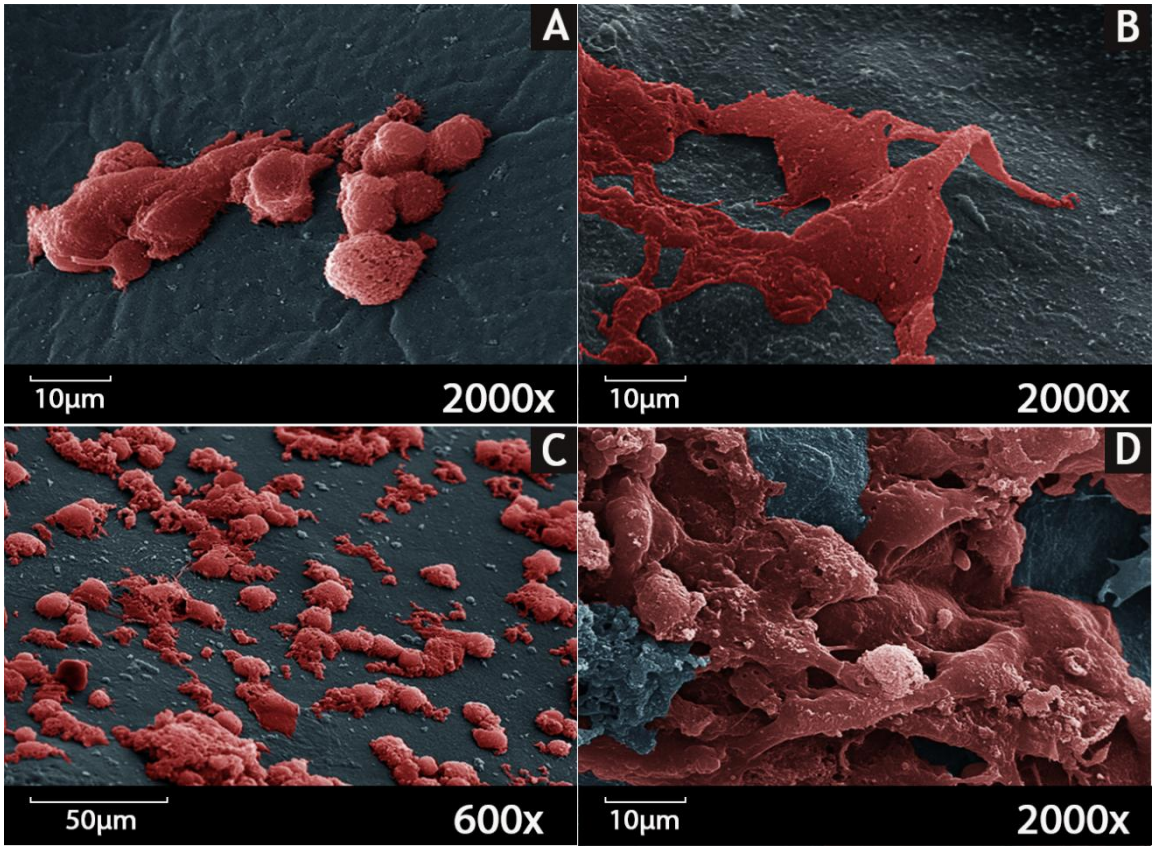


Figure 19: SEM images of human fibroblast cells in contact with CAH (0.75% Chitosan) after 24h, (A) and after 72h, (B). SEM images of human fibroblast cells in contact with CAH (1.5% Chitosan) after 24h, (C) and after 72h, (D).

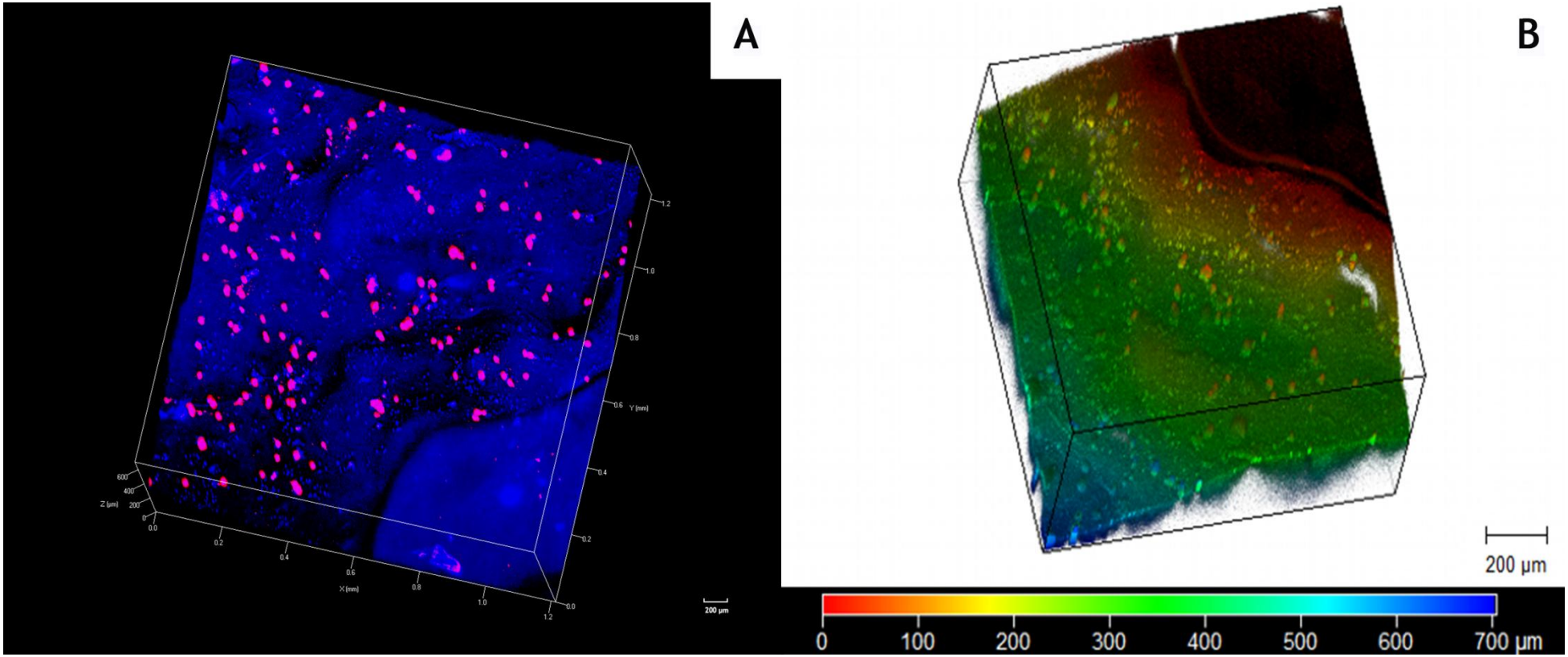


Figure 20: 3D visualisation of the cell localization within CAH (0,75%Ch) using stacked CLSM images (A); 3D visualisation of the cell depth in the CAH (0,75%Ch) using stacked CLSM images and depth coding (B). Scale bar: 200µm (A) an (B).

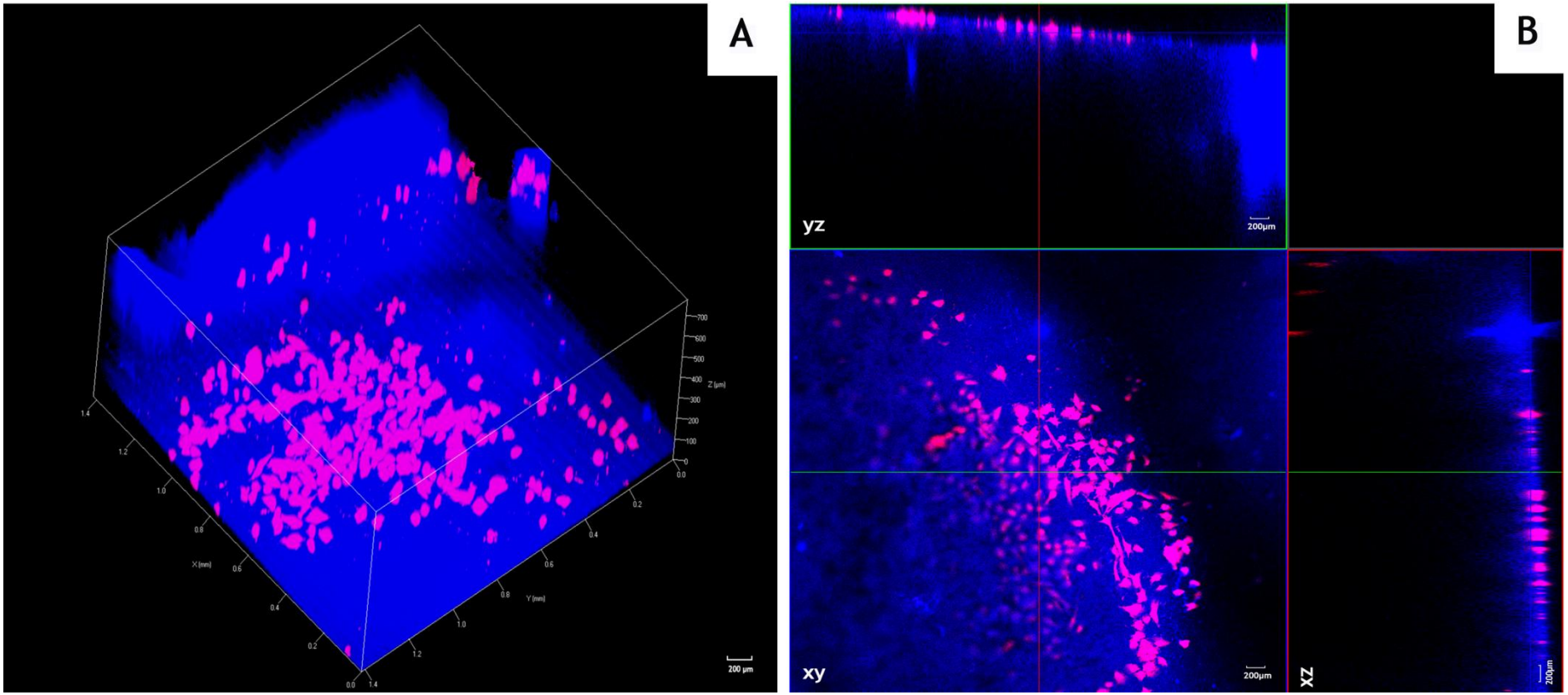


Figure 21: 3D visualisation of the cell internalization in the CAH (1,5%Ch) using stacked CLSM images (A); orthogonal projection of CAH (1,5%Ch) in plan xy, yz and xz (B). Scale bar: 200µm (A) and (B).

3.5 Characterization of the cytotoxic profile of CAH

The cytocompatibility of CAH was characterized through an MTS assay. As already mentioned, human fibroblast cells were seeded in contact with CAH using different concentrations of the agarose and chitosan. Afterwards, the CAH were placed in 96-well plate with DMEM-F12. The cellular viability was assessed over time (at 24h and 72h). Cell viability was quantitatively measured using this MTS non-radioactive assay. In this test a tetrazolium compound is reduced, by living cells, to yield a water-soluble formazan product (Chan *et al.*, 2010).

The MTS assays results (Figure 22) showed that cells in contact with the CAH had higher viability than the positive control, during the period of incubation. It also showed a difference between the positive control ($p<0.01$) and the negative control and cells in contact with CAH. However, the results obtained, for 1% and 2% concentration of agarose, were significantly different between 24 and 72 hours ($p<0.01$), as it can be seen there was a slight decrease in cell viability after 72h. Such, result can be explained by the fact that agarose promote the structural integrity of hydrogel and when the concentration of the agarose is low, the hydrogel degrades along time. Based on this result, we can correlate the concentration of the agarose with the structural integrity of CAH. In the form of a hydrogel, agarose has porous structure and provides a friendly environment for cellular spreading and proliferation. Cell migration is essential for tissue repair and regeneration. The ability of supporting cells to migrate through a hydrogel material guarantees the construction and normal function of the regenerated tissue (Cao *et al.*, 2009).

Moreover, the presence of chitosan in CAH also influences its biological properties. The chitosan provides a non-protein matrix for 3D tissue growth and activates macrophages at wound site. It stimulates cell proliferation and histoarchitectural tissue organization. Chitosan is a haemostat, which helps in natural blood clotting and blocks nerve endings reducing pain. Previous studies have shown that chitin-based dressings can accelerate repair of different tissues facilitate contraction of wounds, and regulate secretion of the inflammatory mediators (Jayakumar *et al.*, 2011).

When the concentration of chitosan increases, there are free amine groups that promote the cell adhesion and proliferation (Dash *et al.*, 2011). However, when the concentration of chitosan increases too much, the aggregates of chitosan precipitate within the agarose matrix, avoiding fibroblasts penetration into CAH (Cao *et al.*, 2009). This fact explains the cell viability observed in the different groups where the concentration of the chitosan is 2,0% and 2,5%. As expected, the positive control (K+) showed no viable cells, as it can be confirmed in figure 22. The results demonstrated that these compounds used to produce the CAH did not affect cell viability and can be used in a near future for skin regeneration.

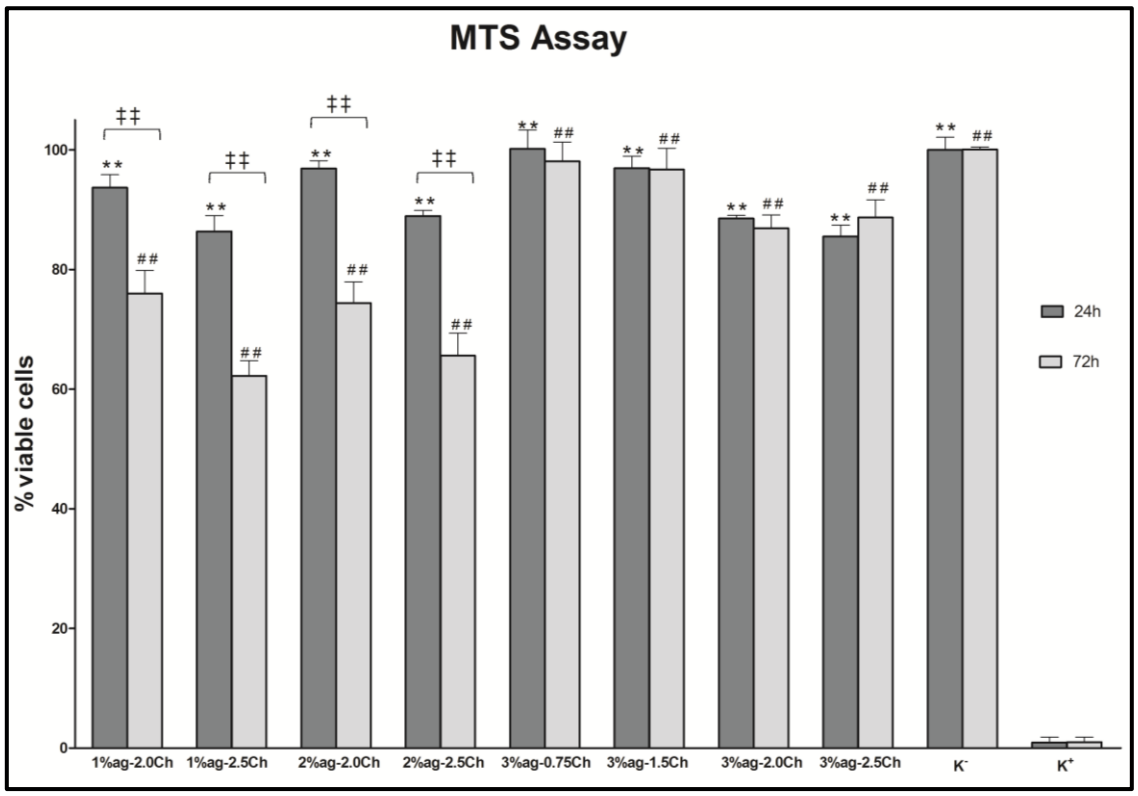


Figure 22: Cellular activities measured by the MTS assay after 24h and 72h. K^+ (death cells); K^-(live cells); ag (agarose); Ch (chitosan). Each result is the mean ± standart error of the mean of at least three independent experiments. Statistical analysis was performed using one-way ANOVA with Dunnet's post hoc test (**, ## ,‡‡$p<0.01$).

3.6 Evaluation of the antimicrobial activity of the CAH

When skin injuries occur, is frequent to observe the invasion of microorganisms at wound site and the bacterial infection becomes a major factor that impairs the skin regeneration. Therefore the control of bacterial multiplication and invasion is of great importance (Jayakumar *et al.*, 2011).

This characteristic is essentially important in the treatment of severe burn wounds, which are easily infected. Sometimes, after implantation of a wound dressing is observed the appearing of infectious organisms at wound site, leading to serious infections that frequently require removal of the wound dressing and excision of cutaneous wounds (Mi *et al.*, 2002). Traditionally, the injured area should be covered with antimicrobial cream once or twice each day (Mi *et al.*, 2002). However, the patients suffer frequently from a considerable of discomfort. Recently, antibacterial wound dressings have been developed in order to inhibit wound infection and improve the typical course of healing with formation of normal granulation tissue, and accelerated angiogenesis (Jayakumar *et al.*, 2011).

The antibacterial properties of the produced CAH were evaluated using one bacterial strain, *Staphylococcus aureus*. The bacterial strain was deemed appropriate for testing the antibacterial properties of the CAH, since it has been reported to be the most common Gram-positive pathogen found in skin infections, associated with the use of biomaterials (Juan *et al.*, 2010).

The antibacterial effects of the hydrogel were evaluate by MIC, which is the standard microbiological procedure used to evaluate the bacteriostatic and bactericidal properties of antimicrobial agents. The MIC value was analysed after 24h of incubation, at 37°C, performing a resazurin assay. This test is based on measuring the metabolic activity of living bacteria by determining the concentration of resorufin, as can be seen in Figure 23A. Resofurin is a pink fluorescent compound produced by reduction of resazurin (7-hydroxy-3H-phenoxazin-3-one-10-oxide) by viable bacteria (Gonzalez and Tarloff, 2001).

Control bactericidal tests of CAH were also performed 24 hours after *Staphyloccocus aureus* be in presence of several amounts of hydrogel (25-100 µl) with concentrations of chitosan between 125-750 µg/mm^3 (Figure 23B). Chitosan concentrations ranging from 281-400 µg/mm^3 were sufficient to decrease bacteria population of *Staphyloccocus aureus*. The results obtained revealed a significant difference between test groups and the positive control ($p<0.01$). Furthermore, the concentration of 563-750 µg/mm^3 had an antimicrobial activity against microorganism, as depicted in Figure 23B.

The chitosan has been found as an attractive candidate in production of antibacterial wound dressings. In general, the various chitosan-based implants evoke a minimal foreign body reaction, with little or no fibrous encapsulation. It observed the typical course of healing with

formation of normal granulation tissue, often with accelerated angiogenesis (Jayakumar *et al.*, 2011, Mi *et al.*, 2002). As previously described, its considerable antibacterial activity against a broad spectrum of bacteria can be explained by the interaction between positively charged chitosan and negatively charged microbial cell wall leads to the leakage of intracellular constituents. Large amount of amino groups are able to enhance the antibacterial activity (Kim *et al.*, 2008).

The MIC value of CAH was about 200 $\mu g/mm^3$, promoting the reduction of bacteria population to <50%, within 24 hours. This concentration of chitosan allowed the interaction between chitosan and the cellular wall of the microorganism, promoting bacterial death. As we expected, the antibacterial activity of CAH improved by increasing chitosan concentration in the gels. More adsorbed chitosan would result in greater changes in the structure of bacterial cell wall and in the permeability in the cell membrane. Both adverse effects result in the death of the bacteria (Chung *et al.*, 2004).

In order to evaluate the formation of the biofilm at CAH surface, SEM images were also acquired with *Staphyloccocus aureus* in contact with hydrogel during 24h (figure 24A and 24B). In the negative control (figure 24C), where the *Staphyloccocus aureus* was placed in agar plate, the formation of the biofilm was visualized.

Two mechanisms have been proposed to explain chitosan's antibacterial activity. In the first, positively charged chitosan reacts with negatively charged molecules at the cell surface, thereby changing cell permeability and avoiding material entrance into cell and/or material being leaked from the cell (Amano, 2009, Helander *et al.*, 2001). The second mechanism involves the binding of chitosan to DNA, to inhibit RNA synthesis. Based on the data reported in the literature it is possible to state that the antibacterial actions of chitosan are a combination of both. As mentioned by others research groups, there are several factors that influence the antibacterial activity of chitosan (Chung *et al.*, 2004, Chung and Chen, 2008).

This hydrogel produced here showed to provide a defense barrier against action of microorganisms and also allowed cell growth, properties that are fundamental for its application in skin regeneration.

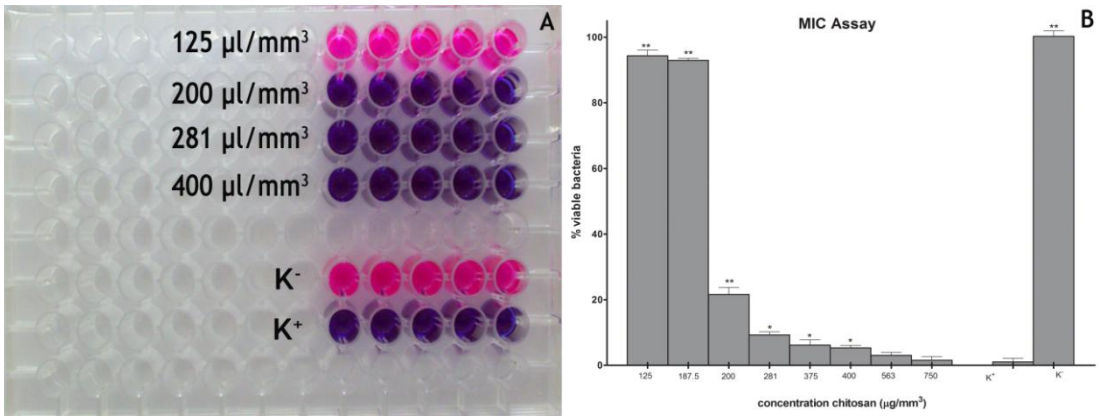


Figure 23: Representation of the reduction of the resazurin by viable bacteria. (A). Blue colour corresponds to the death bacteria and pink to the live bacteria. MIC values of the CAH after 24h of the incubation(B). K^-(live bacteria); K^+ (death bacteria); agarose (ag); chitosan (ch). Each result is the mean ± standart error of the mean of at least three independent experiments. Statistical analysis was performed using one-way ANOVA with Dunnet's post hoc test (*p<0.05; **p<0.01).

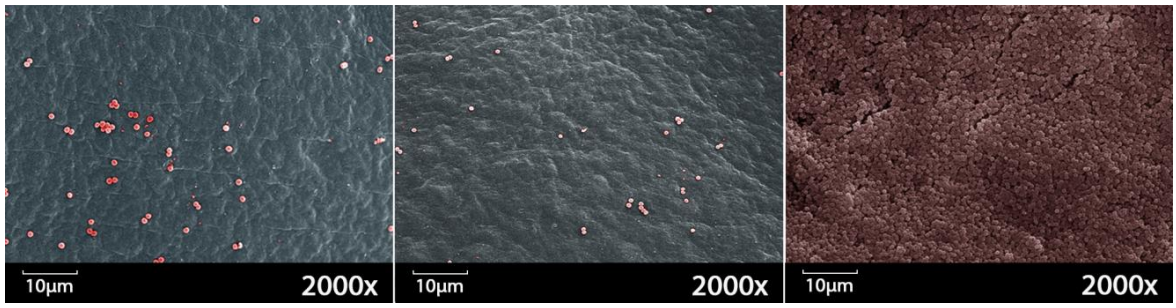


Figure 24: SEM images of *Staphylococcus aureus* in contact with CAH (0.75% Chitosan) after 24h, (A) and in contact with CAH (1.5% Chitosan) after 24, (B). Negative control, (C).

Chapter IV

Conclusion

4. Conclusion

Wound healing is a major worldwide health problem that particularly affects the elderly and diabetic population. In the last years, different therapeutic approaches have been proposed to improve the wound healing process. Among the different wound dressings developed so far, hydrogels that are biomaterials that better mimic the ECM, have emerged as platforms designed to stimulate and to react to specific cellular responses at the molecular level.

These wound dressings not only provide a favourable 3D microenvironment for cell adhesion and proliferation, but also allows gases, nutrients and waste products diffusion. Furthermore, these 3D matrices also confer protection to the wound from possible secondary bacterial infection.

In this study a versatile, non-toxic, thermoresponsive and antibactericidal hydrogel based on the simple blend of two natural biocompatible polysaccharides, was produced. This hydrogel can be formed *in situ* by changing the temperature facilitating its application and improving the patient compliance and comfort. Furthermore, they can also be shaped to the desired form at the injury site, and adhere to tissues during gelification process. Thus promoting the strength of the tissue-hydrogel interaction and providing a favourable structure for cell proliferation.

In CAH, agarose promotes the formation of a consistent 3D structure at the wound site, allowing the production of a supportive scaffold for cell adhesion and proliferation.

The structure of the CAH was characterized by SEM and FTIR analysis. The characterization proved that the blend between polymers was achieved, and that CAH presents an irregular surface and large pores that promote cell adhesion and migration. Moreover, SEM and CLSM analysis of scaffolds seeded with showed that they were able to accommodate proliferate and produce bioactive molecules that promote a faster wound healing process within hydrogel.

Cell viability was also assessed by an MTS assay and the results confirmed that cells remain viable in contact with the CAH. Thereby, we concluded that, based in our *in vitro* studies, the developed CAH is biocompatibility and that it can be used as a wound dressing. Furthermore, the antimicrobial activity of CAH was also assessed by determining the minimum inhibitory concentration and also by evaluating through SEM biofilms formation. The results showed that a chitosan concentration of 200µg/mm^3 allowed the interaction between chitosan and cellular wall of the microorganism, promoting the reduction of bacteria population to <20%, within 24 hours. The SEM images confirmed this antibacterial activity of CAH, since no *Staphylococcus aureus* biofilm was observed on material's surface. Based on these results we can conclude that this hydrogel has suitable properties to be used as skin substitute.

In a near future, *in vivo* assays could be performed in order to evaluate the local and systemic histocompatibility of CAH. Besides, this hydrogel can be lyophilized in order to allow the maintenance of its intrinsic properties during transport worldwide. This hydrogel can also be extended to other research areas and contribute for the regeneration of others tissues of human body.

Chapter V

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Chapter VI

Appendix

6. Appendix

- Ribeiro, M., Morgado, P., Miguel, S., Coutinho, P.and Correia, I.; Dextran based-hydrogel containing chitosan microparticles loaded with growth factors to be used in wound healing. *Materials Science and Engineering: C*. (2013). **33** (5): 2958-2966.

Materials Science and Engineering C 33 (2013) 2958–2966

Contents lists available at SciVerse ScienceDirect

Materials Science and Engineering C

journal homepage: www.elsevier.com/locate/msec

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

M.P. Ribeiro [a,b], P.I. Morgado [a], S.P. Miguel [a], P. Coutinho [a,b], I.J. Correia [a,*]

[a] CICS-UBI, Health Sciences Research Center, Faculty of Health Sciences, University of Beira Interior, Covilhã, Portugal
[b] UDI-IPG, Research Unit for Inland Development, Polytechnic Institute of Guarda, Guarda, Portugal

ARTICLE INFO

Article history:
Received 4 February 2013
Received in revised form 7 March 2013
Accepted 14 March 2013
Available online 24 March 2013

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

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.

1. 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

* Corresponding author at: Av. Infante D. Henrique, 6200-506 Covilhã, Portugal. Tel.: +351 275 329 002; fax: +351 275329099.
E-mail address: icorreia@ubi.pt (I.J. Correia).

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http://dx.doi.org/10.1016/j.msec.2013.03.025

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 β 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.

2. Materials and methods

2.1. 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).

2.2. Methods

2.2.1. Chitosan microparticle 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.

2.2.2. 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 samples [23].

2.2.3. 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.

2.2.4. 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

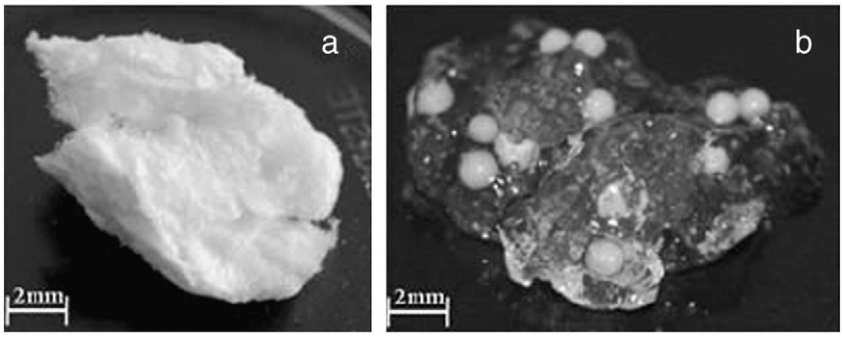

Fig. 1. Images of freeze-drying oxidized dextran (a) and dextran hydrogel with microparticles incorporated (b).

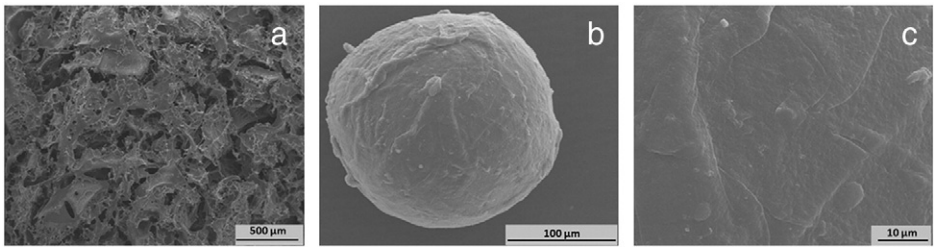

Fig. 2. SEM images of surface of oxidized dextran 50× (a), chitosan microparticles 400× and (b) chitosan microparticles 2000× (c).

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). Lysis solution was added to wells containing cells that were used as positive control (dead cells) [28].

2.2.5. 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].

2.2.6. 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

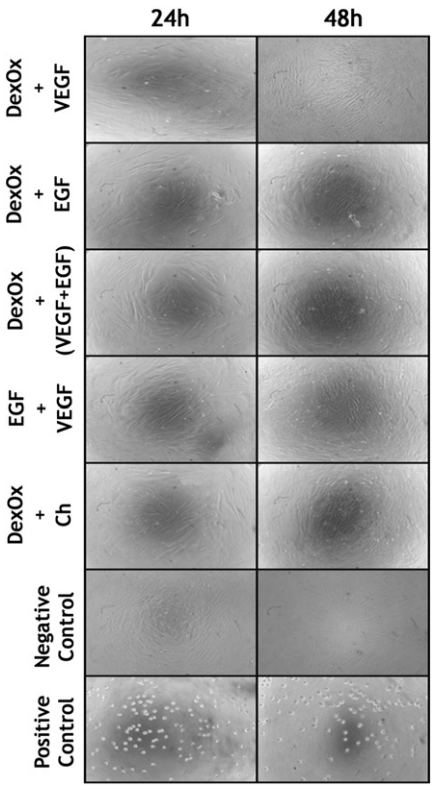

Fig. 3. Microscopic photographs of human fibroblast cells after being seeded in the presence of the carriers during 24 h and 48 h. DeOx + VEGF: oxidized dextran loaded with chitosan microparticles with VEGF incorporated; DeOx + EGF: oxidized dextran loaded with chitosan microparticles with EGF incorporated; 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×.

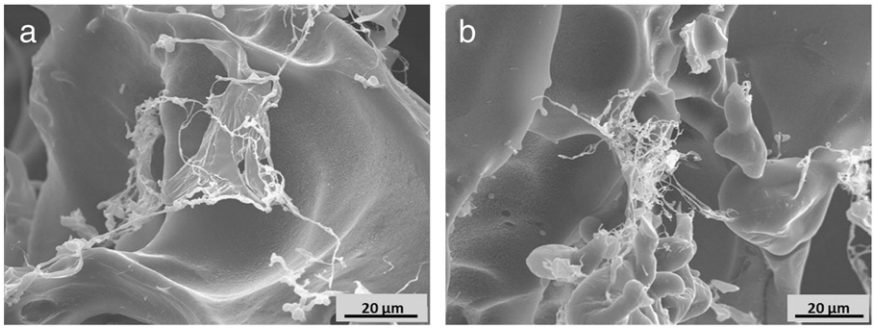

Fig. 4. SEM images of human fibroblast cells in contact with oxidized dextran hydrogel. Original magnification 1000× (a) and oxidized dextran hydrogel with chitosan microparticles incorporated. Original magnification 1000× (b).

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

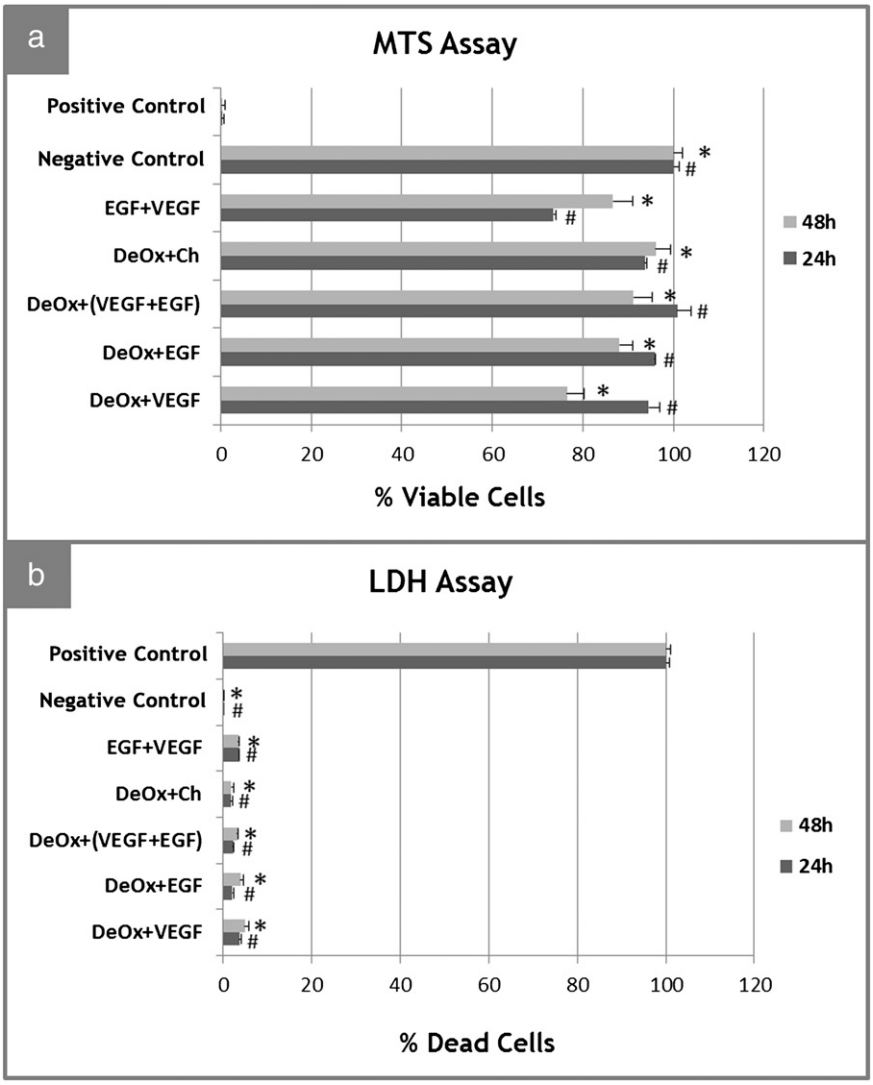

Fig. 5. Cellular activities measured by the MTS assay (a) and cellular integrity measured by the LDH assay (b) after 24 h and 48 h. Positive control (death cells); negative control (live cells); VEGF and EGF (VEGF + EGF); oxidized dextran loaded with chitosan microparticles (DeOx + Ch); oxidized dextran 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 ($^{*}p < 0.05$; $^{\#}p < 0.05$).

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].

2.2.7. 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].

2.2.8. 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):

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

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].

2.2.9. 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.).

3. Results

3.1. Characterization of the morphology of the carriers

Freeze-dried DeOx showed a spongy morphology similar to that of cotton, as can be observed in Fig. 1(a). Subsequently the hydrogel was prepared in Teflon molds and impregnated with microparticles (Fig. 1(b)). SEM analysis of DeOx hydrogel (Fig. 2(a)) revealed a highly porous and interconnected surface. Chitosan microparticles

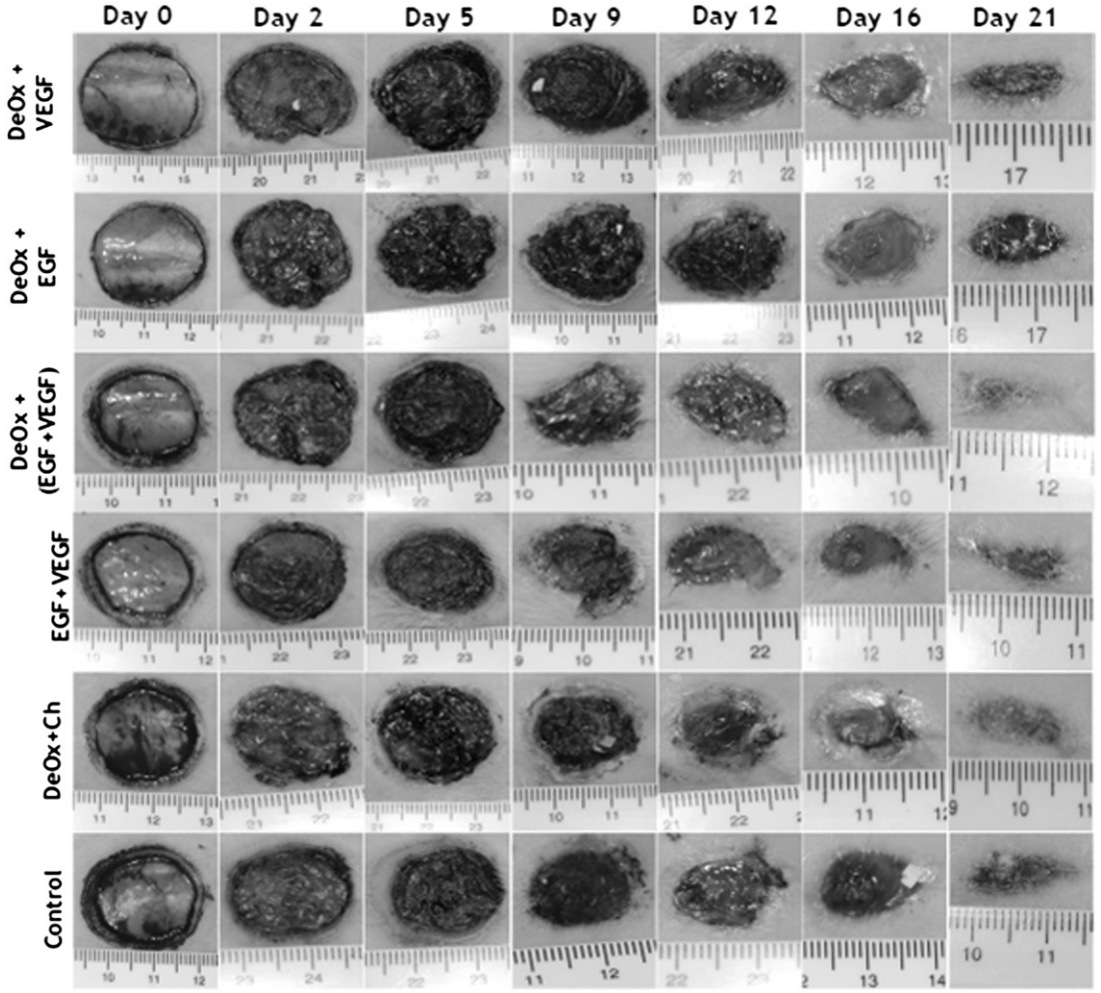

Fig. 6. Macroscopic images of the wound-healing panorama over 21 days. A deep third-degree burn wound with 2 cm diameter was induced at the dorsal skin of each female Wistar rat. Digital images were acquired after the 2nd, 6th, 9th, 12th, 16th and 21st days of the injury.

produced by electrospraying presented a spherical shape and an average diameter of approximately 255 ± 0.9 μm (Fig. 2(b)). Fig. 2(c) shows that the chitosan carriers produced had a slightly smoother surface, in accordance with what was previously reported in the literature [30,31].

3.2. 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 (Fig. 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.

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 hydrogel surface after 48 h (Fig. 4).

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 (Fig. 5). These results clearly demonstrate that these vehicles are biocompatible and may be used for GF delivery systems for wound healing.

3.3. 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 2.2.6. 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]. Fig. 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. In Fig. 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.

3.4. Histological analysis

Fig. 8 presents the histological data obtained in this study. From its observation, it can be concluded that the granulation tissue 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).

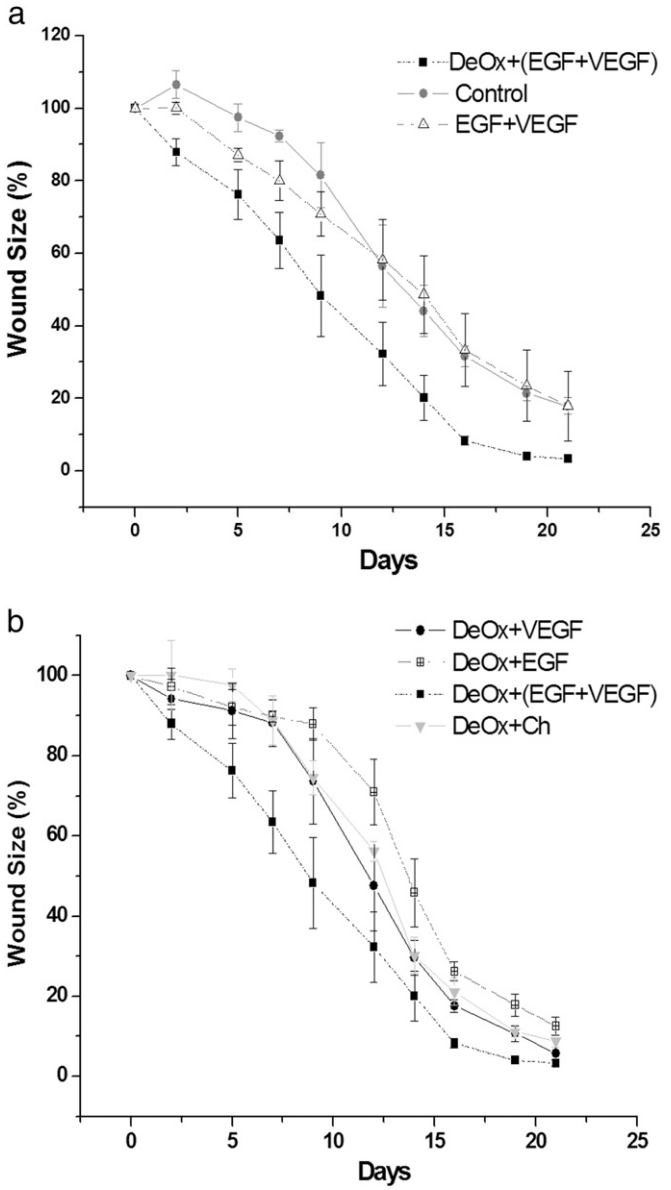

Fig. 7. Effect of DeOx + (EGF + VEGF) on burn wound compared to the control group and EGF + VEGF (a), DeOx + (EGF + VEGF) on burn wound compared to the DeOx + VEGF, DeOx + EGF and DeOx + Ch (b). The surface area of the burn wounds was calculated as described in Methods and reported at each time point 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. 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 the liver, spleen, kidney and colon [8]. Keeping in mind the wound dressing application, the porous section of DeOx (Fig. 2(a)) 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, and local angiogenesis and facilitate nutrient transport, which is fundamental for the wound-healing process [14].

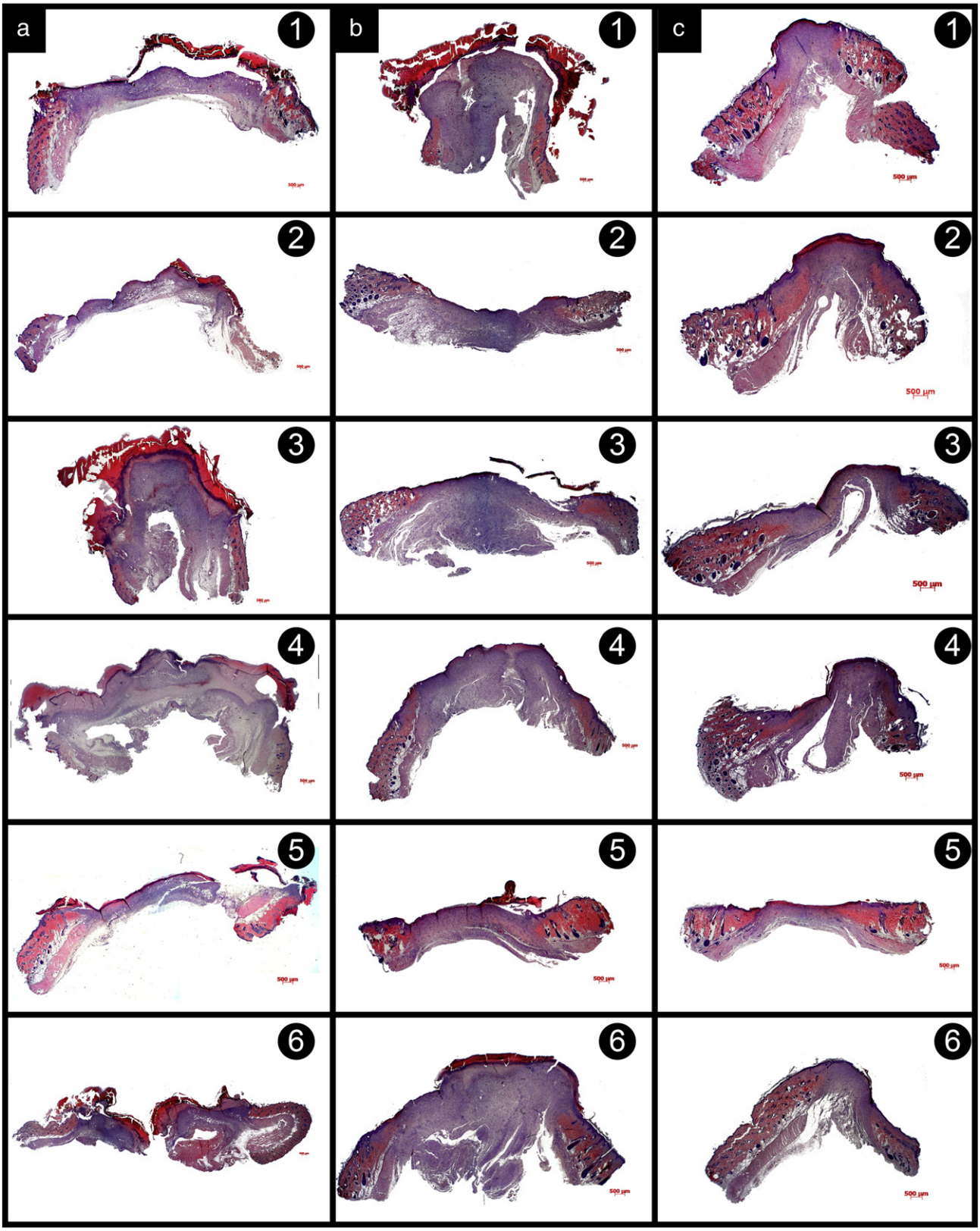

Fig. 8. Hematoxylin and eosin-stained sections of biopsies for the morphological evaluation of skin lesions after 7 days, scale bar 500 µm (a), 14 days, scale bar 500 µm (b) and 21 days, scale bar 500 µm (c). Group 1 treated with EGF + VEGF (1), group 2 treated with DeOx + Ch (2), group 3 treated with 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).

These results are in agreement with experimental data obtained by other researchers [33,34].

In order to increase the period during which GFs were released from the DeOx-based hydrogels, chitosan microparticles loaded with these bioactive molecules were incorporated within 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 (Fig. 2(b)), 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 through 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 dehydration 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 (Fig. 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 rat's skin destroys the 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.

5. 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.

Acknowledgments

The authors would like to thank Ana Paula Gomes for acquiring SEM images and Dr. Catarina Ferreira for her technical support in the preparation of histological samples. The author would also like to thank Fundação para a Ciência e a Tecnologia (FCT) for the financial support (PTDC/EME-TME/103375/2008; PTDC/EBB-BIO/114320/2009; PEst-OE/EGE/UI4056/2011) and the grant awarded to Patrícia Morgado (SFRH/BD/80648/2011).

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