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A novel strategy to produce a drug delivery system for skin regeneration

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Uma nova estratégia para produzir um dispositivo para entrega de fármacos que será usado na regeneração da pele

Diana Patrícia Rodrigues Gaspar

Dissertação para obtenção do Grau de Mestre em

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Those whom I owe my life and who I love forever,

Lucinda and Joaquim…

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Abstract

Skin lesions are traumatic events that lead to the increase of fluid loss, infections, scarring and locally immunocompromised regions. These injuries can be caused by genetic disorders, acute trauma or even surgical interventions. In these situations, a substantial area of skin can be damaged, often without the possibility of being regenerated. Scientists have put a lot of effort in the development of suitable drug delivery systems suitable to release therapeutic molecules that are required for the initials phases of the wound healing process. Cell microencapsulation arises as an alternative approach for sustained *in situ* cell delivery. This technology is based on the immobilization of cells within a polymeric matrix, surrounded by a semi-permeable membrane, that isolate the encapsulated cells from the host immune system. Nonstanding, the microparticulate matrix still allows the exchange of nutrients, gases, waste and releasing of bioactive molecules, such as extracellular matrix components and growth factors secreted by cells. Nevertheless, the optimization of cell-based therapy demands the development of alternative strategies to improve cell administration. Alginate has been used for cell microencapsulation, due to its simple gelling process, excellent biocompatibility, biodegradability properties and its stability under *in vivo* conditions. On the other hand, nanoparticulate systems have been widely used in the biomedical field, as drug delivery devices that can improve the efficiency and widening the applications of the microencapsulation systems. Therefore, the present study aimed to develop biodegradable alginate microparticles that were used for human fibroblasts cells and chitosan nanoparticles encapsulation, in order to improve the wound healing process. To do so, two types of microparticles were firstly produced with alginate and a mixture of alginate and collagen. Subsequently, these carriers were characterized according to their size and geometry by scanning electron microscopy. Confocal images were also acquired to confirm cell encapsulation in microparticles. The cytotoxic profile of the carriers was assessed. Cell release from microparticles was observed over time after encapsulation through optical microscopic analysis. In second part of the work, chitosan nanoparticles loaded with a model protein (bovine serum albumin) were produced and were incorporated in microparticles. The encapsulation efficiency of this protein in nanoparticles was determined. Then, both the morphology and size of these nanoparticles were characterized. The results herein obtained showed that the developed microparticles and nanoparticles can be used as systems tailored for sustainable cells and drug release.

Keywords

Alginate, cell encapsulation, chitosan, microparticles, nanoparticles

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. Estas feridas podem ser causadas por desordens de origem genética, traumas ou mesmo devido a cirurgias. Deste modo, uma área substancial da pele pode ser danificada, muitas vezes sem a possibilidade de regeneração. Os investigadores têm procurado desenvolver novos sistemas de entrega de drogas, de forma a acelerar o processo de cicatrização. O microencapsulamento celular surgiu recentemente como uma nova abordagem, para entrega controlada e de longa duração de agentes terapêuticos produzidos e secretados pelas próprias células, tais como componentes da matriz extracelular e factores de crescimento, os quais são essenciais para a regeneração. Esta tecnologia tem por base a imobilização de células, dentro de uma matriz polimérica rodeada por uma membrana semi-permeável. Assim, as células não são reconhecidas pelo sistema imunitário do hospedeiro e a membrana permite a difusão de nutrientes e gases para o interior da matriz e a saída das moléculas bioactivas secretadas pelas células e dos resíduos resultantes do metabolismo celular. No entanto, a terapia celular necessita ainda de ser optimizada. O alginato é um polímero que tem sido usado para o encapsulamento celular, devido ao seu fácil processo de gelificação, excelente biocompatibilidade, biodegradabilidade e estabilidade *in vivo*. Por outro lado, os sistemas nanoparticulados têm sido amplamente utilizados em aplicações biomédicas, por exemplo na produção de dispositivos de entrega direcionada de moléculas bioactivas, uma vez que permitem obter um perfil de libertação controlado. O presente trabalho teve como objectivo o desenvolvimento de micropartículas de alginato para encapsular fibroblastos humanos e nanopartículas de quitosano, com o intuito de futuramente serem usadas como agentes promotores da cicatrização de feridas. Inicialmente, foram produzidos dois tipos de micropartículas, um à base de alginato e outro de alginato com colagénio. As micropartículas produzidas foram caracterizadas quanto ao seu tamanho e geometria por microscopia electrónica de varrimento. Posteriormente, foram também adquiridas imagens de confocal para confirmar o encapsulamento de células nas micropartículas. O perfil citotóxico dos transportadores foi caracterizado através de testes de viabilidade celular, os quais confirmaram a biocompatibilidade dos transportadores. O perfil de libertação das células foi observado por análise microscópica ao longo dos dias. Numa segunda parte do trabalho foram produzidas nanopartículas de quitosano com o objetivo de serem incorporadas nas micropartículas como transportadores de factores de crescimento e, assim, favorecer a cicatrização das feridas. A eficiência de encapsulação das nanopartículas foi avaliada através da incorporação de uma proteína modelo, albumina de soro bovino. Posteriormente fez-se a caracterização da morfologia e do tamanho destas nanopartículas. Os estudos efectuados demonstraram que o sistema desenvolvido é adequado para a libertação de células e moléculas bioativas de forma controlada, prolongada e em concentrações fisiológicas.

Palavras-Chave

Alginato, encapsulamento de células, micropartículas, nanopartículas, quitosano

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List of Abbreviations

2D	Two-dimensional
3D	Three-dimensional
aFGF	Acid fibroblast growth factor
BCA	Bicinchoninic acid
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
CH_3COOH	Acetic acid
CLSM	Confocal laser microscopy
DD	Degree of deacetylation
DMEM	Dulbecco's modified Eagle's medium
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EtOH	Ethanol
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
GAGs	Glycosaminoglycans
GF(s)	Growth factor(s)
HA	Hyaluronic Acid
HMW	High molecular weight
K^-	Negative control
K^+	Positive control
KGF	Keratinocyte growth factor
MMP	Metalloproteinases
MTS	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium
NaOH	Sodium hydroxide
PBS	Phosphate-buffered saline

PEG	Polyethylene glycol
PGA	Polyglycol acid
PHEMA	Poly (hydroxylethyl methacrylate)
pI	Isoelectric point
PLA	Polylactic acid
PLGA	Poly (lactic-co-glycol) acid
PMS	Phenazine methosulfate
PVA	Poly (vinyl alcohol)
SEM	Scanning electron microscopy
TGF-β	Transforming growth factor beta
TIMP	Tissue inhibitors of metalloproteinase
TPP	Pentasodium tripolyphosphate
UV-VIS	Ultraviolet-visible
VEGF	Vascular endothelial growth factor

Chapter I:

INTRODUCTION

1.1. Skin

1.1.1. Functions and Structure

Skin is considered the largest organ of the human body, covering about 1.7m^2 and comprising approximately 8% of the total body mass [1-4].

Skin serves, primarily, as a protective and defensive barrier against the external environment and allows the maintenance of the body temperature. This organ is also involved in defensive mechanisms, which confer physical, immunological, metabolic and UV radiation-protective barriers against microbes and toxic chemicals. Moreover, skin can also be used as a entry point for therapeutic substances such as vaccines [1,3,5,6]. Skin importance is demonstrated by the fact that when large areas of this tissue are lost, caused by diseases or injuries (as in the case of extensive burns), result in significant morbidity and mortality [3,7].

Skin is organized in three anatomical distinct layers known as the epidermis, dermis and hypodermis [3,8,9] (figure 1). These layers have different thickness, strength and flexibility [8]. The epidermis is the most superficial layer of the skin and provides the first barrier to avoid invasion of foreign substances into the body. Keratinocytes and melanocytes are prominent cell types of this layer. The main function of the dermis is to support the structural integrity of the skin giving it durability and elasticity. Such properties are confered by the major cellular components of the dermis, the fibroblasts. The hypodermis contains mostly adipose tissue, which functions as a thermal insulator and also helps to protect the underlying structures [3]. Skin appendages like hair follicles, sebaceous and sweat glands are founded in the dermis and epidermis [9].

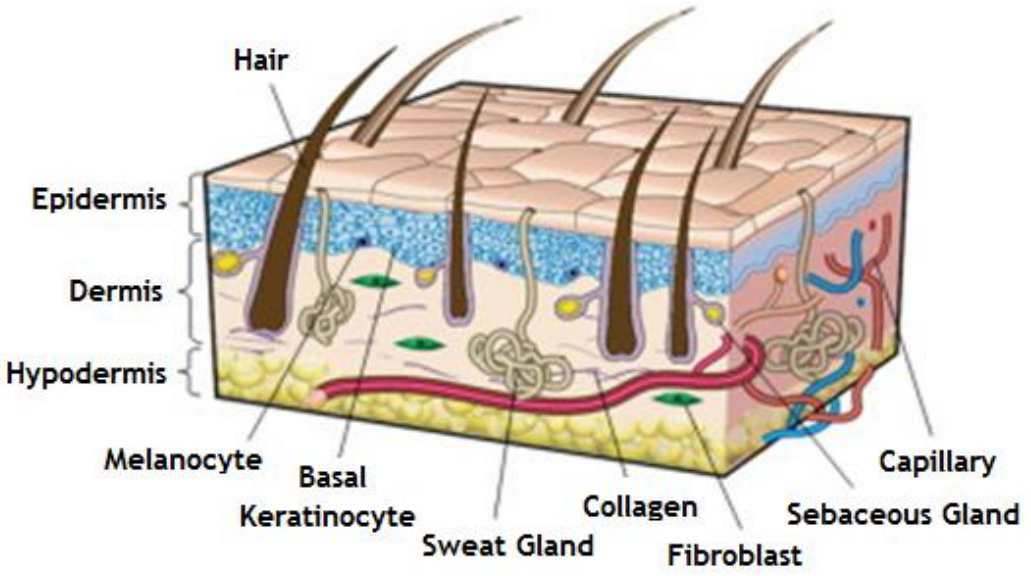


Figure 1: Representation of the structure of the human skin (adapted from [9]).

1.1.2. Epidermis

The epidermis is the superficial layer of the skin and serves as an impermeable boundary between the environment and the body. This sheet is a multilayered cellular structure with little or no extracellular matrix (ECM) and do not present blood vessels [4,8].

Besides keratinocytes, which account for about 80% of epidermal cells, the epidermis is also composed of the pigment-producing cells, the melanocytes and specialized dendritic Langerhans cells, that have an essential role in the skin immune defense system [8].

The epidermis may be subdivided, from the inside to the outside, into five layers: the strata basale, spinosum, granulosum, lucidum and corneum [4].

The continuous proliferation, differentiation and, ultimately, cell death allows compartmentalization into a number of strata representing different stages of keratinocyte maturation [8]. The keratinocyte cell division starts in the innermost strata basale and pushes daughter cells apically towards the next spinosum strata, where there are more cells. As they progress toward the skin surface, they move into the granulosum strata, where they accumulate lipid granules, critical for the maintenance of the water barrier [8].

Pigmentation is imparted by melanin, which is produced by melanocytes and transferred to keratinocytes in the final sublayer of the strata lucidum. The most external layer, known as the stratum corneum is formed by completely differentiated dead keratinocytes, interspersed with intercellular lipids (mainly ceramides and sphingolipids) [8].

The basement membrane allows the separation of the epidermis from the dermis [3]. This membrane has many functions, among which it is responsible for epidermis binding to the dermis. It also determines polarity of the epidermis and provides a barrier against epidermal migration, which prevents the direct contact of epidermal cells with the dermis [10].

1.1.3. Dermis

The dermis is the layer between epidermis and hypodermis. It contains a high number of fibroblast cells that produce collagen and elastin, which are the main constituents of the ECM and give support and elasticity to the skin, respectively [3,11].

This layer is divided in two zones: a lower layer called reticular and a superficial one nominated papillary. The deeper layer, which is the main component of the dermis, is continuous with hypodermis and consists of dense irregular connective tissue. This tissue gives strength and elasticity to skin [3,12,13]. On the other hand, the papillary layer contains extensions nominated papillae, which are projected to the epidermis [13].

The dermis contains blood capillaries, which supply the epidermis and help to regulate body temperature, smooth muscle fibers, sweat and sebaceous glands and their ducts, hair follicles and sensory nerve endings [13].

1.1.4. Hypodermis

The hypodermis is the bottom layer of the skin that is located beneath the dermis and it is involved in the connection between skin, bones and adjacent muscles [14].

This layer is well vascularized and is composed mainly of adipose tissue, where about half of the total stored fat in the body is localized [13,14]. However, their amount vary with age, sex and feeding [13]. Thus, hypodermis confers insulation, acts as an energy source and also contributes to the mechanical properties of the skin [9,14].

Nevertheless, some authors do not contemplate hypodermis as being part of the skin, considering it as a subcutaneous tissue [13].

1.2. Wounds

Wounds affect millions of people worldwide, rendering this pathology one of the major issues in modern health care [15,16]. A wound can be described as a disruption of the structural or functional integrity of the skin, that is caused by physical or a thermal damage. Moreover, they can also result from an underlying medical or physiological condition [3,17,18].

Based on the type of repair process, wounds can be classified as acute or chronic wounds. Acute wounds are usually characterized by a complete healing, with minimal scar formation. The primary causes of acute wounds include mechanical injuries due to external factors, such as abrasions and tears, that are caused by frictional contact between the skin and hard surfaces. Other acute wounds include burns and chemical injuries, which arise from a variety of sources such as radiation, electricity, corrosive chemicals and thermal sources [17]. The chronic wounds arise from tissue injuries that heal slowly or fail to heal due to repeated tissue insults or underlying physiological conditions, like diabetes, persistent infections and poor primary treatment. Such is responsible for the disruption of the events sequences, that occur during the wound healing process [17].

Wounds are also classified based on the number of layers and area of skin affected. Therefore, they can be divided into epidermal, superficial partial-thickness, deep partial-thickness and full-thickness, with increasing depth of the injury [17,19]. Epidermal injuries caused by sunburns, light scalds or grazing are characterized by erythema and minor pain. These injuries do not require specific surgical treatment, since only the epidermis is affected and it regenerates rapidly without scarring [19].

Superficial partial-thickness wounds affect the epidermis and superficial parts of the dermis. This type of wounds is characterized by epidermal blistering and severe pain, especially in the case of thermal trauma. The blood vessels, sweat glands and hair follicles are affected [17,19].

Deep partial-thickness injuries involve a great dermal damage that results in fewer skin appendages (such as hair follicles, sweat and sebaceous glands) and therefore they take longer to heal [19].

Full-thickness injuries are characterized by the complete destruction of the underlying subcutaneous fat or deeper tissues, in addition to the epidermis and dermal layers [17,19].

Except for very superficial wounds and in early fetal life, the regenerative capacity of the skin is limited and in the majority of the wounds occurs the formation of high amounts of scar tissue [3].

1.2.1. The Wound Healing Process

The wound healing is a complex biological process that involves growth and tissue regeneration, in order to prevent organism deregulation. After an injury, multiple biological pathways are immediately activated in a variety of cellular and matrix components that act together to reestablish the integrity of the damaged tissue and replace the injured tissue [15,17,18,20-23].

Wound healing comprises five overlapping stages that involve complex biochemical and cellular processes (see figure 2). These are described as haemostasis, inflammation, migration, proliferation and maturation phases [15,17,20-23].

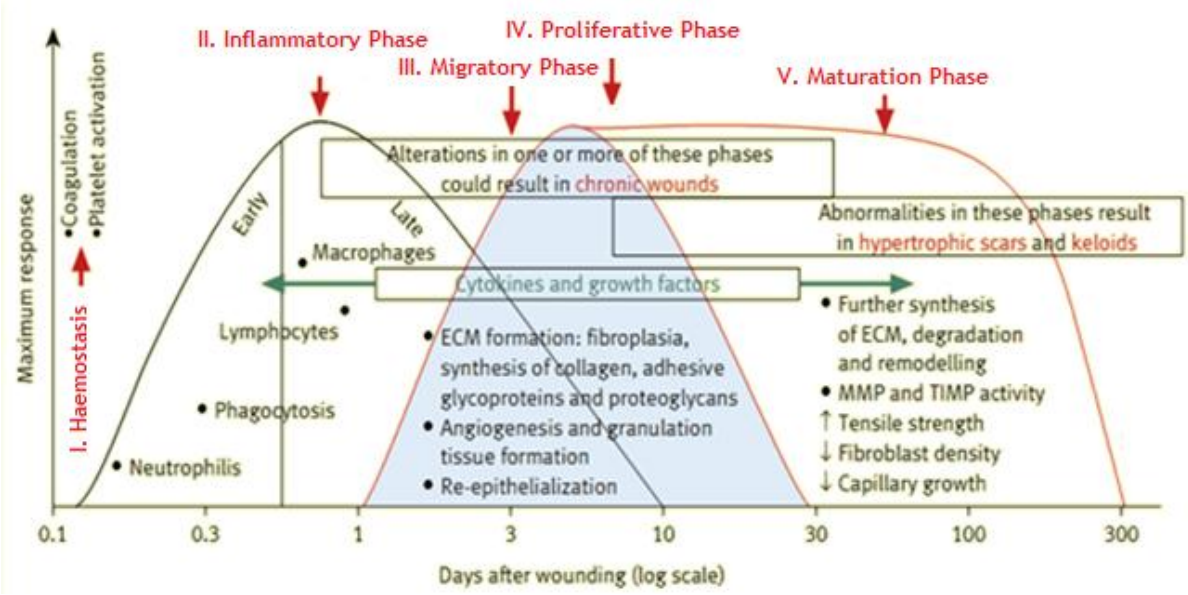


Figure 2: Phases of the wound healing process. This procedure occurs in a cascade of events that is correlated with the appearance of different cell types in the wound, during various stages of the healing process (ECM: Extracellular matrix; MMP: Metalloproteinases; TIMP: Tissue inhibitors of metalloproteinases) (adapted from [18]).

1.2.1.1. Haemostasis and Inflammation

Haemostasis occurs immediately after tissue damage due to the onset of bleeding [3,17]. Components of the coagulation cascade, inflammatory pathways and immune system (e.g. neutrophils, monocytes and lymphocytes) are needed at the wound site to prevent ongoing fluid losses [18,24]. Haemostasis is achieved initially by the formation of a platelet plug, followed by the formation of a fibrin matrix, that becomes the scaffold for infiltrating cells. The clot dries to form a scab and provides strength and support to the injured tissue [15,17,22].

Inflammatory phase occurs simultaneously, involving both cellular and vascular responses. The main purpose of this phase is to clean the wound by removing dead tissues and to

prevent the occurrence of infection [3]. Thus, in this phase, neutrophils and platelets are abundant at the injured site [15]. The release of protein-rich exudate into the wound causes vasodilation through the release of histamine and serotonin, and allows phagocytes to enter to the wound, surrounding the dead cells. Platelets released from damaged blood vessels become activated as they enter in contact with mature collagen and form aggregates as part of the clotting mechanism (figure 3) [15,17,22].

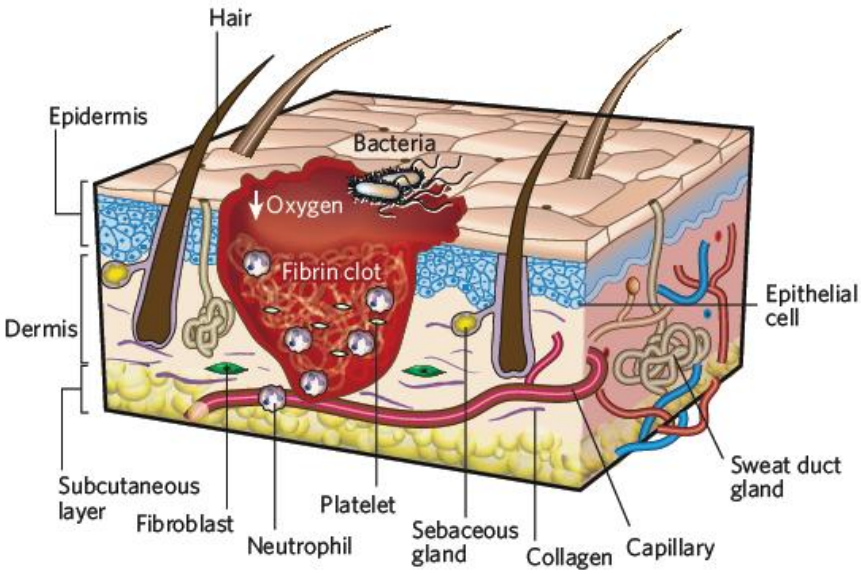


Figure 3: Representation of inflammatory phase of the wound healing process (adapted from [15]).

1.2.1.2. Migratory Phase

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. These cells regenerate from the margins (figure 4), rapidly growing over the wound under the clot accompanied by epithelial thickening [15,17].

1.2.1.3. Proliferative Phase

The proliferative phase occurs almost simultaneously or just after the migration phase and is characterized by fibroblast migration, deposition of ECM and formation of granulation tissue. This tissue is formed by the in-growth of capillaries and lymphatic vessels into the wound. In this phase occurs also the formation of blood vessels, which is known as angiogenesis, as depicted in figure 4. The fibroblast proliferation and collagen synthesis occurs each time edema recedes [3,15,17,18,22,24].

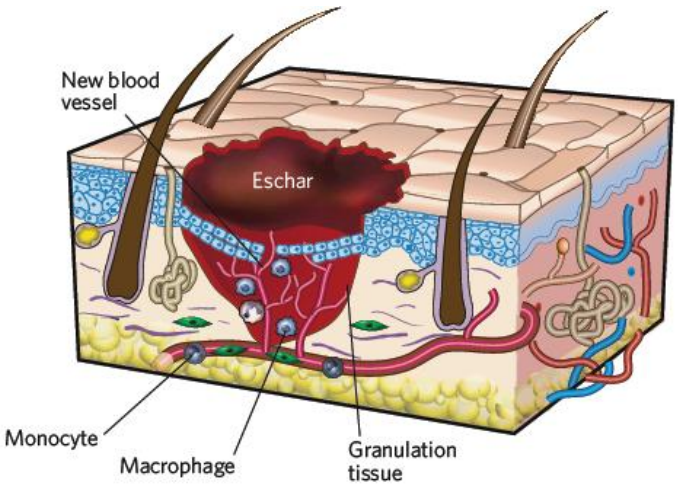


Figure 4: Illustration of migratory and proliferative phases of the wound healing process. A scab was formed on the surface of the wound (adapted from [15]).

1.2.1.4. Maturation Phase

During the maturation or remodeling phase, all the processes that are activated after the injury ceases (figure 5). The majority of endothelial cells, macrophages and myofibroblasts undergo apoptosis or exit from the wound, leaving a mass that contains few cells and consists mainly of collagen and other ECM proteins. This stage involves the formation of cellular connective tissue and strengthening of the new epithelium. Such determines the nature of the final scar. Moreover, the acellular matrix is mainly composed of collagen type III that will be replaced by collagen type I, later on. This process is carried out by matrix metalloproteinases that are secreted by fibroblasts, macrophages and endothelial cells [3,15,17,24]. The re-epithelialized wound surface is slightly higher than the surrounding surface, and the healed region does not presents any skin appendages [15].

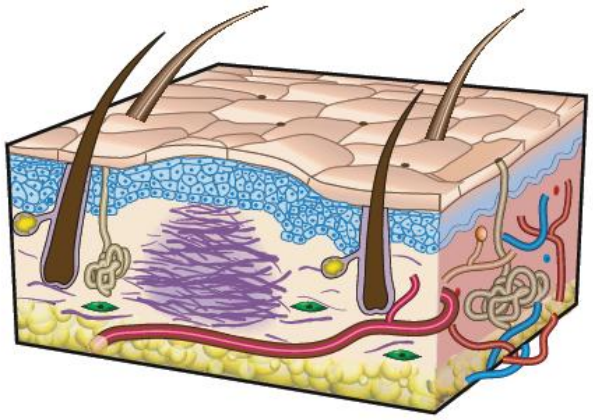


Figure 5: Representation of the maturation phase of the wound healing process (adapted from [15]).

1.3. Tissue Engineering: Skin Substitutes

There are a number of pathologies in which skin loss or damage occurs. Among them, burns and chronic ulcers are responsible for the loss of superficial epidermis and dermis, abnormal wound healing and the failure of (or delay associated with) wound healing process [9,16]. The healing of a skin wound is a complicated phenomenon that includes a wide range of cellular, molecular, physiological and biological processes. The immediate coverage of the wound with a dressing is a cornerstone of wound management [6].

In the past, the application of dressing materials on top of a wound was aimed for bleeding inhibition, protection of the wound from the pathogens (e.g. bacterias), as well as avoid water and electrolyte disturbances [17,20]. However, traditional dressings do not restrict moisture evaporation and may cause dehydration of the wound bed [25].

With the purpose to overcome such problems, grafts have been used for deep partial- and full-thickness wounds treatment [6]. Tissues for skin grafting can be obtained from the self-patient (autografts), from a different person of the same specie (allografts) or from animals of different species (xenografts) [26]. The characteristics of autografts are ideal, since the patient does not present any immunological rejection. Although autografts exhibit the best clinical outcome, they suffer from several disadvantages such as the limited quantity, donor site morbidity and the requirement of surgery at multiple sites to harvest donor tissue from the patient [7,27,28]. Allografts and xenografts have distinct advantages for the treatment of skin defects, especially due to the unlimited tissue quantity availability. However, these type of grafts are associated with the risk of disease transmission and require the use of immunosuppressants with associated side effects, which can lead to graft rejection [2,23,27,29,30]. Therefore, these practices have significant limitations for the patient thus, not accomplish the purpose of the skin recovery [23,29,31].

In order to abolish the limitations of autografts, allografts and xenografts, different studies have been performed in the area of tissue engineering. This is a multidisciplinary area of knowledge, in which new three-dimensional (3D) scaffolds with adequate properties are developed to allow cell attachment, migration, proliferation and differentiation until the newly formed tissue is structurally stabilized [32]. These biomaterials support tissue regeneration that had suffered some type of injury. Therefore, its purpose is to recreate living, healthy and functional tissues. [32,33]. These scaffolds are also usually employed to create an immune-protected environment that allows nutrient, gases and waste products diffusion required for cells survival [34].

Therefore, a huge effort has been done in the area of tissue engineering to improve the wound healing mechanism. The different skin substitutes have been developed in order to avoid

the need to remove the skin's patient from one part to another and decrease the patients' risk of infection and sepsis. Also, they contribute to reduce the mortality and morbidity caused by scarring, changes in pigmentation, the total number of surgical procedures and hospitalization period [35,36] . The concern for the development of skin substitutes is also suitable for extensive wounds that cannot heal spontaneously, since they affect a high percentage of total body surface area or for injuries that cannot heal due to inflammation or a deficiency wound healing process. These substitutes can also be used to accelerate the healing of wounds that would heal by themselves, however very slowly [36].

Skin substitutes should act as scaffolds for cells that are used to replace lost tissue rather than just facilitate the process of wound healing [6,17,35,37]. These skin substitutes try to mimic the normal physiologic responses, during the wound healing and function as coating to protect the wound against infection, while allowing gaseous exchanges [17,38]. Consequently, an ideal skin substitute must also protect the injury from fluid and proteins loss, be easy to handle and apply to the wound site, undergo controlled degradation, enable exudates remotion, inhibit exogenous microorganism invasion (antimicrobial properties) and improve aesthetic appearance of the wound site [6,27,39-41]. It should also be non-toxic, non-allergenic, be easily removed without trauma, and it should be made from a readily cheap and available biomaterial, that requires minimal processing.

Presently there are a lot of skin substitutes that have been developed. They can be classified based on: 1) the anatomical structure that they reproduce (dermo-epidermal (composite), epidermal and dermal); 2) the period among which they are used (permanent, semi-permanent or temporary); 3) the origin of the material used to produce the biomaterial (biological, synthetic and semi-synthetic) and 4) cellular or acellular components [28].

Dermo-epidermal or composite skin substitutes aim to mimic the structure of normal skin where both epidermal and dermal layers are injured. Most of these skin substitutes have fibroblast and keratinocytes incorporated into a scaffold, in order to form a temporary covering. These systems act rather like temporary biologically active wound dressings, providing growth factors (GFs), cytokines and a ECM for host cells, while initiating and regulating wound healing process [9,19]. The keratinocytes usually used in this type of skin substitute provide pain relief and accelerate wound healing [6], however they do not survive longer than few weeks when applied to the wound. Allograft™, Apligraft™ and OrCel™ are examples of current commercially available dermo-epidermal skin substitutes [9,19].

Epidermal substitutes usually contain autologous keratinocytes that are isolated by skin biopsy. The use of autologous keratinocytes in this type of skin substitute is advantageous, since the risk of rejection is very low. However, these skin substitutes present also some disadvantages, such as long preparation time, variable engraftment rates, difficult handling due to their thickness, fragile cellular layers and high production costs. Epicel™, MySkin™ and

CellSpray™ are some of the commercially available epidermal substitutes that have been approved for clinical use [9,19].

Engineered dermal substitutes were designed and produced to restore dermal tissue by promoting new tissue growth and optimizing healing conditions. However, they need to be covered by a permanent epidermal surface. The majority of products for dermal substitutions are composed by acellular matrices. After their application, they are colonized and vascularized by the underlying cells, producing an autologous neo-dermis. Others skin substitutes have human cells incorporated and are applied as transient wound dressings that stimulate the healing mechanism. In Dermagraft™, fibroblasts are seeded in a mesh, where they secrete GFs, deposit dermal matrix proteins and thus improve the healing process. It is much easier to manufacture these types of skin substitutes when compared with cell-containing bilayered skin substitute constructs. AlloDerm™ and Karoderm™ are others examples of the commercially available dermal bioengineering constructs [9,19].

Some of the developed tissue engineered products and skin substitutes presently available are described in Table 1.

Table 1: Skin Substitutes Available for Clinical Use.

Type	Name of Dressing	Major Components	Refs
Epidermal and Dermal Skin Substitutes	Integra™	A bovine collagen based dermal analogue and a thin temporary epidermal silicone sheet	9,17,42
	Biobrane™	A nylon mesh that is bonded to a silicone membrane and coated with collagen	17,42
	TranCyte™	A polyglycolic acid (PGA)/polylactic acid (PLA) layer with ECM proteins	17
	Apligraft™	Bovine type I collagen mixed with a suspension of allogeneic fibroblasts and keratinocytes	9,11,14,17
Dermal Skin Substitute	Alloderm™	Allograft human dermis with all the cellular material removed	9,17
	Dermagraft™	Cultured human allogeneic fibroblasts on a biodegradable PGA or polyglactin mesh	9,14,17
Epidermal Skin Substitute	Epicel™	Cultured autologous human keratinocytes which are grown to stratified cell sheets	9,17
	MySkin™	Cultured autologous human keratinocytes on a silicone polymer substrate	9,11,14,17
	Hyalograft 3-D™	Autologous human fibroblasts incorporated on a laser-microperforated hyaluronate	17
	Laserskin™	Autologous human keratinocytes cultured on a membrane of benzyl hyaluronate	14,17
	Bioseed™	Fibrin sealant and cultured of autologous human keratinocytes	17

Scientists have been developed other types of engineering skin substitutes with others natural and synthetic materials. Examples of natural materials include polypeptides, hydroxyapatites, hyaluronic acid (HA), glycosaminoglycans (GAGs), fibronectin, collagen, chitosan and alginates. Such materials have the advantage of presenting low toxicity and a low inflammatory response [43]. Examples of synthetic materials include polyglycolide, polylactide and polylactide coglycolide, which are used for sutures and meshes. However, there are other synthetic materials like polytetrafluoroethylene and polyethylene terephthalate that are also used to fabricate skin substitutes [43]. Nevertheless, there is still a remaining gap to be filled, since no engineering skin substitute used until now, presents all the criteria needed for fully restore the functional skin [23,43].

The ultimate goal of tissue engineering of the skin is to rapidly create a construct that offers the complete regeneration of functional skin, including all the skin appendages (such as hair follicles, sweat glands and sensory organs) and layers (epidermis, dermis and fatty subcutus) with rapid vascularization and the establishment of a functional vascular and nerve network and scar-free integration with the surrounding host tissue [43]. These advances are depicted in figure 6.

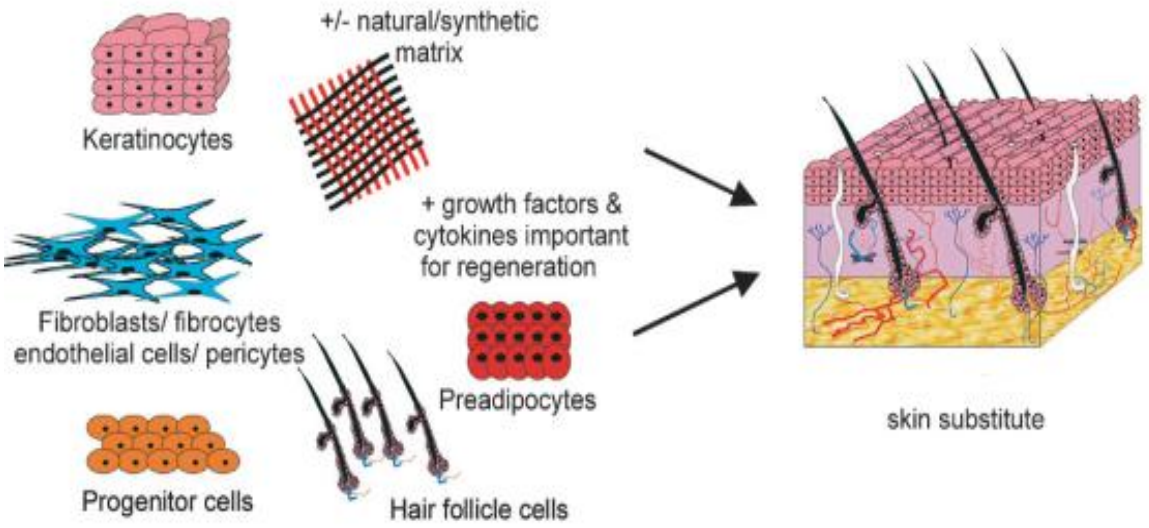


Figure 6: A schematic of the requirements to create a fully functional skin substitute (adapted from [14]).

In this new generation of skin substitutes, engineered scaffolds either produced with natural or synthetic materials will also be potentially useful for the delivery of additional signal molecules, such as GFs and nucleic acids to the wound site. This procedure could help in speeding up cell migration, adhesion, proliferation and differentiation, allowing the formation of granulation tissue and re-epithelization in a shorter period [6,17]. The use of a combination of these signaling molecules not only improve the tissue repair, but also regulates and promotes the *in*

vitro tissue growth [6,7,38]. Other approach to design these skin substitutes are to mimic the functions of the ECM components naturally found in tissues [32].

1.3.1. Cell Encapsulation as a Strategy for Skin Regeneration

Cell therapy is one of the most exciting fields in regenerative medicine. It aims to replace, repair or enhance the function of damaged tissues or organs [44]. Recently, cell therapy emerged as a promising strategy in skin tissue engineering, since it is a viable alternative for improving and restoring biological function of tissues [32,45].

Encapsulation of living cells in spherical-shaped devices (microbeads or microparticles) has been used for the continuous delivery of drugs and cells to treat various health disorders, such as endocrine (diabetes, hypoparathyroidism), central-nervous diseases (Parkinson's and Alzheimer's) and cancer [12,46-53].

This technology is based upon the entrapment of cells within a polymeric matrix surrounded by a semi-permeable membrane for the long-term release of therapeutics molecules [54]. The cells produce and secrete the bioactive agents continuously to the outside matrix, while the semi-permeable membrane isolate the cells from the host immune system preventing the recognition of the immobilization cells as a foreign material (e.g., antibodies and cytokines), and allowing the exchange of nutrients, gases and waste [44,47,48,50,54-56]. These features are depicted in figure 7. On the other hand, the polymeric matrix structure and chemistry must be suitable to allow cell survival and tissue formation, while its degradation rate should be similar to that of tissue growth. Finally, the degradation products must not be toxic for the encapsulated cells [33].

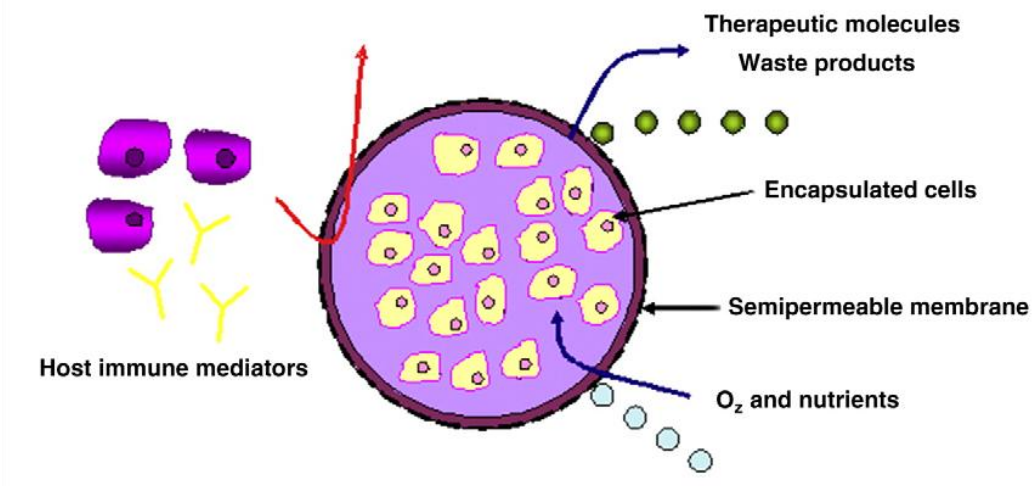


Figure 7: Representation of an encapsulation system. It is expected to allow nutrient, gases and GFs diffusion, processes that are essential for cell survival (adapted from [44]).

Cell immobilization shows important advantages compared with the encapsulation of proteins or other bioactive molecules, since it allows a continuous release of the therapeutic molecules in physiological concentrations [44,54]. Furthermore, it also allows the delivery of cells into the defect area and to support the cellular functions during the early phases of the regeneration process [57]. Therefore, cell encapsulation is one of the primary techniques of tissue engineering to delivery cells to the injured site, without being dependent on cells' ability to migrate to the defect area [58].

One of the most important considerations for the design and production of a cell microencapsulation device is the material biocompatibility that is used. Moreover, these materials can not affect the surrounding host tissue, neither interfere with cell homeostasis [59]. Table 2 presents several examples of recent materials that have been used for cell encapsulation [33].

Table 2: Materials used for cell encapsulation.

Material	Degradation Mechanism	Cells Encapsulated	Refs
Chitosan	Enzymatic (lysozyme)	Chondrocytes or cardiomyocites	33
Alginate-co-Gelatin	Hydrolytic	Hepatocytes	12,33
HA	Enzymatic (hyaluronidase)	Chondrocytes, fibroblasts, human mesenchymal stem cells	12,33
Chondroitin Sulfate	Enzymatic (chondroitinase)	Chondrocytes	33
PEGylated Fibrinogen	Enzymatic (plasmin)	Bone marrow stromal cells	33
Self-assembled Peptide Gels	Dissolution	Human mesenchymal stem cells, preosteoblasts, endothelial cells, embryonic stem cells	33
Elastin-like Polypeptide	Hydrolytic	Chondrocytes, osteoblasts, adipose-derived stem cells	12,33
Poly (ethylene glycol) (PEG) based	Enzymatic (lipase) or hydrolytic	Chondrocytes	33
Polyfumarate based	Hydrolytic	Bone marrow stromal cells	33
Phosphoester	Hydrolytic	Mesenchymal stem cells	33

1.3.1.1. Hydrogels used for Cell Encapsulation

Hydrogels are a class of hydrophilic biomaterials that have been used for cell encapsulation, with therapeutic purposes. The hydrogel structure allows cell encapsulation within a 3D environment, similar to that of the natural ECM of soft tissues, thus allowing good mass transport to maintain cell viability [12,60,61]. Hydrogels can absorb the wound exudates and have cross-linked networks that have high water contents and tissue-like elastic properties. These attributes make them ideal candidates to be used in tissue engineering, especially for wound healing [21,33].

The design and production of a suitable hydrogel for cell encapsulation, must meet several criteria. In a typical cell microencapsulation process (figure 8), cells are suspended in a liquid precursor solution prior to encapsulation. The process by which hydrogel gelation or solidification occurs must be mild and cell friendly. The hydrogel structure and chemistry must be suitable for cell survival and tissue formation, while its degradation must occur in a timescale comparable to that of tissue regeneration [62]. Finally, the degradation products of the hydrogel must not have adverse effects for the encapsulated cells [33]. Generally, the encapsulation process can occur through a change in temperature for thermal sensitive gels (e.g., agarose, collagen, or gelatin), or via ion-gelation (e.g., alginate) and photo-based cross-linking mechanism (e.g., PEG) [63].

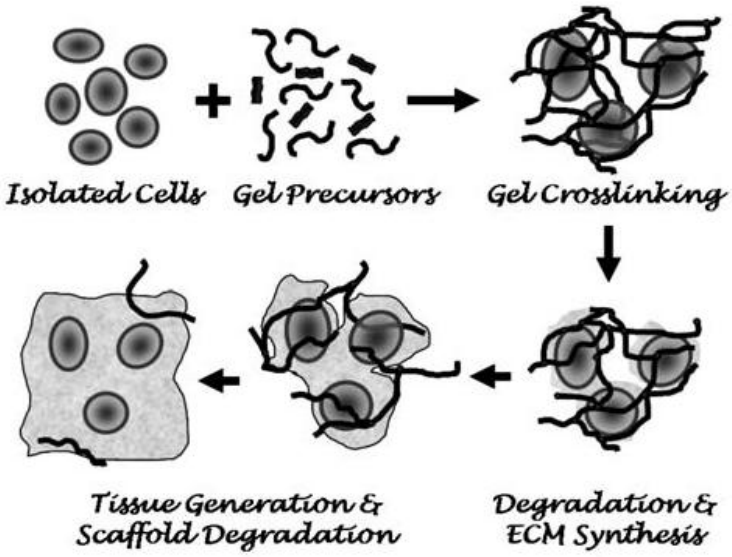


Figure 8: Representation of the process of cell encapsulation. This strategy involves the mixture of cells with precursors, in a liquid solution followed by a gelation process (adapted from [33]).

Hydrogels used for cell microencapsulation are commonly processed into spherical beads, which in size range from 100 to 2000 μm [64]. Hydrogel microparticles have been developed for cell encapsulation, due to their spherical shape, which is considered advantageous from a mass transport perspective, offering an optimal surface-to-volume ratio for protein, nutrient and cells

diffusion [44,62]. Spherical appearance is increasingly recognized as a valuable tool in tissue engineering, since the microparticles appear to mimic the morphology and physiology of the cells in living tissues and organs better than conventional, two-dimensional (2D) monolayer cultures [65].

They also provide a highly hydrated microenvironment for embedded cells that can present biochemical, cellular, and physical stimuli that guide cellular processes such as differentiation, proliferation, and migration [44]. They can also induce cell–cell and cell-ECM interactions within the bulk of the material [66]. Moreover, these microcarriers provide a high degree of permeability for low-molecular mass nutrients and metabolites [21,44,62].

The versatility of these systems is associated with the variables that can be modeled to obtain an optimal system for a specific application, such as the particle composition, size, shape, existence of porosity, cell culture conditions, surface topography and chemistry or incorporation of bioactive agents [67].

Cell microencapsulation devices can be classified into 3 categories (figure 9): 1) matrix-core/shell microcapsules; 2) liquid-core/shell microcapsules and 3) cells-core/shell microcapsules.

In matrix-core/shell microcapsules, the cells are hydrogel-embedded, which has several advantages as the cells are kept in an aqueous environment, in contact with a soft and biocompatible material. Liquid-core/shell microcapsules generally seem to allow better cell growth and protein production, although their mechanical resistance is lower than that of the matrix-core capsules. In cell-core/shell microcapsules, the objective is to produce thinner membranes, which optimize cell diffusion [62].

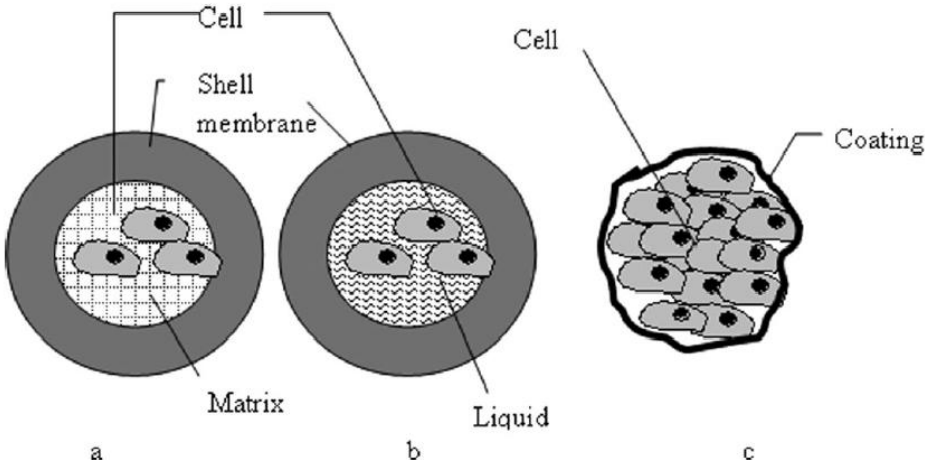


Figure 9: Classification of the different microencapsulation devices. (a) Matrix-core/shell microcapsules (cells embeded in hydrogel matrix); (b) Liquid-core/shell microcapsules (cells suspended in liquid core); (c) Cells-core/shell microcapsules (direct cell coating) [62].

Hydrogels can be produced with natural materials, that can replicate the physiological characteristics of native tissue [66]. Polysaccharides (e.g., agarose, gelatin and HA) and natural ECM proteins (e.g., collagen, gelatin and fibrin) have been used for the production of hydrogels [45,66].

Synthetic polymers have also been used in regenerative medicine to produce hydrogels using various molecules such as polycaprolactone, poly (lactic-co-glycolic acid) (PLGA), PEG and its derivatives, poly (vinyl alcohol) (PVA), polyurethane, poly (hydroxylethyl methacrylate) (PHEMA) and others. The mechanical and chemical properties of synthetic polymers can be tailored for different applications without having the immunogenicity-related concerns of some naturally occurring polymers, which constitutes the main advantage of this type of materials over the natural ones [45,66].

1.3.2. Alginate as a Material for Cell Encapsulation

As previously described in the text, different materials have been employed for cell encapsulation. Among them, alginate is nowadays one of the most studied and characterized materials due to its excellent biocompatibility and stability under *in vivo* conditions [68].

Alginate is a linear copolymer of (1–4)-linked ß-D-mannuronic acid (M-block) and α-L-guluronic acid (G-block) (figure 10). It is a naturally occurring polysaccharide extracted from brown seaweed and bacterium and its composition vary depending upon the source from which it is isolated. Both cell adhesion and hydrogel stiffness can be influenced by these two uronic acid salts ratio [7,12,31,60,69,70]. However, only the G-block of alginate is believed to participate in intermolecular cross-linking with divalent cations (e.g., calcium, barium, and strontium) to form 3D structures. Calcium chloride ($CaCl_{2)}$ is the most frequently used cross-linking agent to reticulate alginate [54,68,70-72].

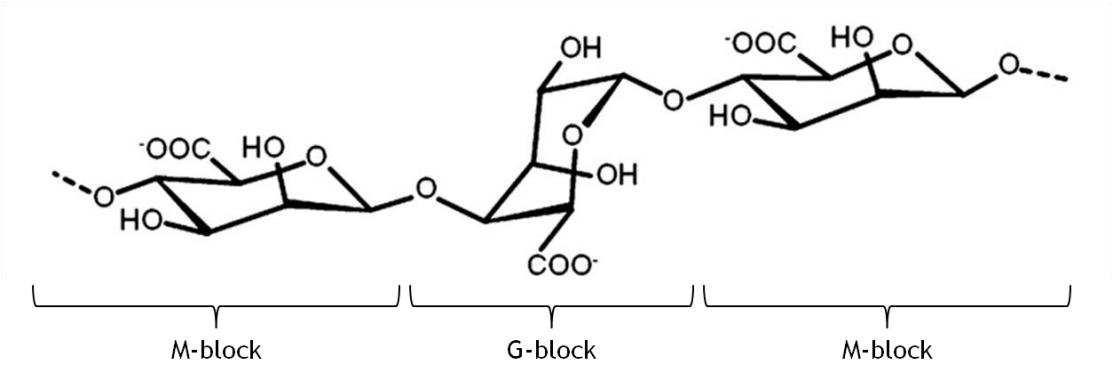


Figure 10: Chemical structure of sodium alginate (adapted from [71]).

Because of its low toxicity, abundance, mechanical integrity/stability and easy gelling properties, alginate hydrogel has been investigated for a variety of tissue engineering applications, particularly in cell encapsulation [7,31,60,71]. In addition, it does not interfere with the cellular function of the immobilized cells in alginate gels and finally the encapsulation procedure can be conducted under very mild conditions, such as body temperature and at physiological pH [34,72].

The biocompatibility presented by this polymer is essential for the production of devices that protect the enclosed cells from the host immune system [44,73]. Other important feature is that its hydrated 3D network allows cells to adhere, spread, migrate and interact with each other. Moreover, cell encapsulation in alginate is a flexible technology and some parameters may be adjusted (i.e., alginate concentration, alginate composition, microparticle size and cell seeding density) to meet specific cell culture parameters [74].

However, the gelling reaction of alginate can be reversed by removing the calcium cross-linker through the exposure of the carrier to a number of other ions like sodium. Such mechanism leads to the scaffold degradation *in vivo* [31,60,71]. Therefore, under *in vivo* conditions and in order to have a successful tissue engineering approach, the rate of scaffold degradation should be similar to that of tissue regeneration [31].

Alginate has also been used for wound dressings production, and it is known to promote wound healing mechanism [71]. When in contact with the lesion bed, alginate, as a gel, is easily removed from the injury, decreasing pain and trauma associated with the dressing change procedure [1]. Furthermore, it provides a moist environment that accelerates tissue granulation formation, re-epithelialization [1], and minimize bacterial infection at the wound site [71]. Alginate interferes with the normal pro-inflammatory response to bacterial infection given by macrophages, decreasing the time of clearance of pathogenic organisms from the organism [75].

1.4. Nanoparticles

An appropriate delivery carrier of biomolecules is very critical in the area of drug delivery, pharmaceutics, tissue engineering and even stem cell research [76]. For drug delivery purposes, nano-systems, ranging in size from 10-1000nm, have been developed to act as a reservoir of therapeutic agents, with spatial and temporal control release profiles of the drug, leading to desirable therapeutic outcomes [77]. In recent years, a significant effort has been devoted to develop drug delivery nanodevices for treating various diseases, such as cancer [77].

Different types of nano-sized carriers, such as polymeric nanoparticles, solid lipid nanoparticles, ceramic nanoparticles, magnetic nanoparticles, polymeric micelles, polymer-drug conjugates, nanotubes, nanowires, nanocages and dendrimers have been developed for various drug delivery applications [78].

Both natural and synthetic materials can be used to produce these different nanoparticulate delivery systems [77]. During this last decade many of biodegradable polymers such as polysaccharides (e.g., alginate, chitosan), polyesters (PLA, PGA and polycaprolactone) have been used to produce devices for protein and drug delivery. Among them, the polysaccharides and their derivatives have been used in different studies [79,80]. Nanoencapsulation of drugs, proteins, or other molecules within these biodegradable polymers has been recognized as an effective way for producing a nanoparticle that can hold target biomolecules and do its delivery [76].

Despite the variety of nanoparticles, these nanodevices must have some general characteristics, such as the ability to incorporate the drug without loss of activity, tunable release kinetics, stability when applied *in vivo*, biocompatibility, lack of immunogenicity, degradability and the potential to reach specific organs and tissues [77]. Nanocarriers may also protect a drug from modify drug tissue distribution profile and/or improve intracellular penetration and distribution [78].

The nanoparticles, due to their small size, can penetrate through smaller capillaries and be taken by cells, allowing an efficient drug accumulation at the target sites [53].

These nanosystems can be prepared by various methods such as chemical cross-linking, ionic cross-linking, emulsion droplet coalescence, reverse micellar and self assembly chemical modification [81,82]. Drug loading into nanoparticulate systems can be done during the preparation of particles (incorporation) and after the formation of particles by incubating drug into the solution [82-84]. In these systems, drug is physically embedded into the matrix or adsorbed onto its surface [82]. On the other hand, drug release from nanoparticles is determined by the diffusion of drug molecules through the polymer network and/or material degradation [77].

Recently, it has emerged the need to control and to prolong the release profile of the drugs incorporated into the nanoparticles. In some cases (as pulmonary administration),

nanoparticles are also impractical for management owed to the fact that their direct delivery poses many challenges, including formulation instability due to particle-particle interactions and poor delivery efficiency due to low-inertia nanoparticles [85]. To overcome such obstacles, it has arisen the hypothesis to incorporate these nanodevices in microparticles. Thus, this microencapsulation technique allows a better retention of bioactive molecules in the system preventing them from being released and increasing the period among which they are released [77,85].

1.4.1. Nanoparticles for Growth Factors Delivery

The immobilization of GFs in 3D matrices has already been studied by several investigators [86]. GFs are proteins that induce a change in cellular function by transducing proliferation or differentiation signals and are involved in the modulation of tissue growth and development [87,88].

Topical administration of GF is still a challenging mission, since proteins present a short half-life *in vivo*, side effects caused by multiple or high doses applied and protein denaturation, which are some of the barriers for a successful GF based therapy. Most, if not all, GFs that are administered in their native form and without any protection are susceptible to be degraded or be rapidly eliminated from the blood circulation, resulting in insufficient amounts of these proteins to play their function in the body [88]. Different methods have been tested to overcome these disadvantages. The encapsulation of a GF in a delivery system has been demonstrated to be a good strategy for GFs-based therapeutics [88].

Owing to their ultra-small size, nanoparticle formulations have many advantages over the traditional dosage forms used for GFs delivery. They are able to enhance the action of these active agents at the treatment sites for a sufficient period of time, in order to allow the cells to migrate, proliferate and differentiate at the injury. They also increase the systemic circulation lifetime and then release them in a sustained and controlled manner [53,89]. These delivery systems also minimize the release of GFs to non-target sites, and support tissue regeneration that normally occurs over long periods of time. Therefore, this direct GF delivery technology is crucial to deliver key exogenous signalling proteins needed for the developing of new tissues [88]. Encapsulating GFs within or attaching them to a polymer nanocarrier can remarkably improve its safety, efficacy and enable the development of new therapies [88].

In skin, GFs are involved in the maintenance of homeostasis during wound healing [90], because they stimulate endogenous repair mechanisms by providing the right signals to cells and thereby leading to an accelerated functional skin restoration [88]. In wounds, the topical application of several GFs to stimulate fibroblast and endothelial cell proliferation has been used

for heal the impaired wound, in order to increase the granulation tissue and capillary formation, and thus stimulate the wound healing mechanism [91].

Among the different GFs, particularly important factors for skin regeneration include fibroblast GF (FGF) family and epidermal GF (EGF), keratinocyte GF (KGF), vascular endothelial GF (VEGF) and transforming GF beta (TGF-β). VEGF functions as an endothelial cell mitogen, chemotactic agent and inducer of vascular permeability [92]. VEGF is also important due to its effects on multiple components of the wound-healing cascade, including angiogenesis and recently has been shown its role in epithelialization and collagen deposition [92]. Other GFs that positively regulate re-epithelialization include members of the FGF family such as basic FGF (bFGF), EGF and TGF-β [15]. The clinical use of EGF has also been extensively investigated in wound healing processes, especially in the treatment of diabetic ulcers [53]. Both bFGF and acidic FGF (aFGF) have many biological activities that stimulate the proliferation of fibroblasts and capillary endothelial cells, thus promoting angiogenesis and wound healing [91].

1.4.2. Chitosan as a Polymer for Drug Delivery

Chitosan is a natural biodegradable, biocompatible, bioadhesive and bacteriostatic polymer and it gained a lot of attention in the pharmaceutical field, since it has been used for the production of a wide range of controlled drug delivery systems [81,93]. This polymer is extracted and purified from the shells of shrimp, crab and other crustaceans, and from some of the fungi cell walls [94].

Chitosan is a copolymer of glucosamine and N-acetyl glucosamine linked by β(1–4) glucosidic bonds obtained by N-deacetylation of chitin [81,93,94]. The content of glucosamine is called as the degree of deacetylation (DD). Depending on the source and preparation procedure, its molecular weight may range from 300 to over 1000kD with a DD from 30% to 95% [6]. The molecular weight and DD can be modified during its preparation to obtain different properties [81,93,94]. Highly deacetilated forms (DD>85%) exhibit a relatively low degradation rate and may last for several months *in vivo*, whereas the forms with lower DD are degraded more rapidly [6]. These basic molecular parameters of chitosan influence the protein loading and delivery [93]. Different DD and molecular weight of chitosan have been used for the production of micro/nanoparticles [82].

In its crystalline form, chitosan is normally insoluble in aqueous solution above pH 7, however, in dilute acids (pH<6.5), the protonated free amino groups on glucosamine facilitate solubility of the molecule [6]. Chitosan is polycationic in acidic media (pKa 6.5) and can interact with negatively charged species, such as pentasodium tripolyphosphate (TPP) and sodium sulfate, as depicted in figure 11. This characteristic can be employed to prepare cross-linked chitosan nanoparticles and microparticles, that can be used for protein and vaccine delivery [93].

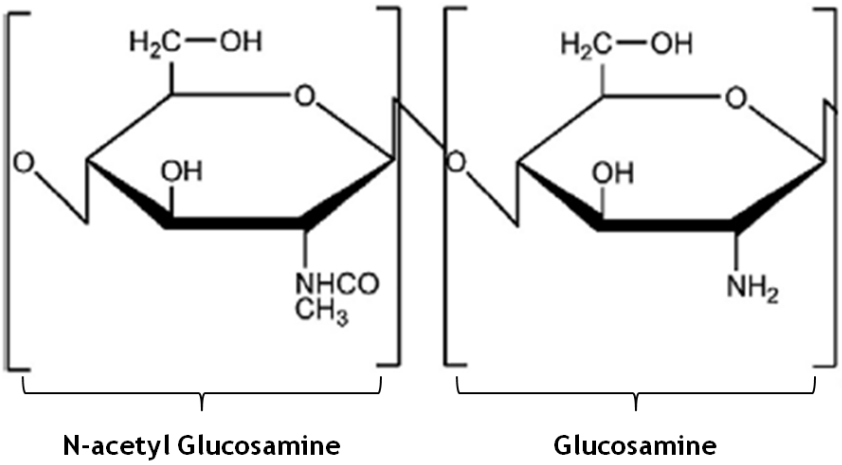


Figure 11: Structure of chitosan (adapted from [95]).

Several studies reported the use of chitosan for the production of skin substitutes [17,23]. This polymer presents different properties that promote the wound healing mechanism, since it has an homeostatic effect and stimulates the synthesis of collagen by fibroblast. Furthermore, it also promotes the infiltration of polymorphonuclear cells at the wound site, which is an essential event for wound healing takes place. Recently, it was also reported that chitosan was used for the incorporation of different GFs, such as bFGF, to improve the rate of healing [6].

1.5. Objectives

In the present study, a new microencapsulation system for future application in skin regeneration had been developed. The main objectives of this study were:

- Development of alginate microparticles with human fibroblasts cells encapsulated;
- Evaluation and characterization of the physical and biological properties of the produced vehicles through spectroscopic techniques;
- Development of chitosan nanoparticles loaded with a model protein, bovine serum albumin (BSA);
- Characterization of the produced nanoparticles through scanning electron microscopy (SEM) analysis.

Chapter II:

MATERIALS and METHODS

2.1. Materials

Acetic acid (CH_3COOH), amphotericin B, BSA, $CaCl_2$, high molecular weight (HMW) chitosan, collagen solution from calf skin, Dulbecco's modified Eagle's medium (DMEM-F12), ethylenediaminetetraacetic acid (EDTA), L-glutamine, penicillin G, TPP, phosphate-buffered saline (PBS), sodium alginate, sodium hydroxide (NaOH), streptomycin and trypsin were purchased from Sigma-Aldrich (Portugal). (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphofenyl)-2H-tetrazolium) (MTS) was obtained from Promega (Madison,WI, USA). Pierce BCA® protein assay reagent A and B were purchased from Thermo Scientific (Portugal). Human Fibroblast Cells (Normal Human Dermal Fibroblasts adult, criopreserved cells) were purchased from PromoCell (Spain). Fetal bovine serum (FBS) was purchased from Biochrom AG (Germany). Hoechst 33342® was obtained from Invitrogen (Carlsbad, CA, USA).

2.2. Methods

2.2.1. Encapsulation of Human Fibroblasts Cells in Alginate

Human fibroblasts cells were encapsulated into microparticles by gelifying alginate microcarriers with calcium ions [79]. For this purpose, a solution of sodium alginate was prepared with milli-Q water. Subsequently, human fibroblasts cells were harvested, counted and resuspended in the alginate solution to a final ratio of 71.8×10^5 cells/mL. The final moisture with a concentration of 1.5% (w/v) was vortexed to ensure a complete mixing of the cells within the solution. The suspension was loaded into a syringe and extruded using a syringe pump (KdScientific, KDS, Sigma), through a 25G needle into a 10mL HEPES buffered $CaCl_2$ (5% (w/v), pH 7.4) solution, under magnetic stirring, for 10 min, at room temperature. This gelling agent was prepared by dissolving $CaCl_2$ in milli-Q water. Droplets produced immediately microparticles due to the ionic cross-linking of alginate matrix with divalent cations. The produced microparticles were then recovered by filtration using a 0.22μm filter paper and then washed three times with DMEM-F12 [31,47,48,60,76,96]. Other type of microparticles composed of collagen and alginate were also produced under the same procedure. The process is represented in figure 12.

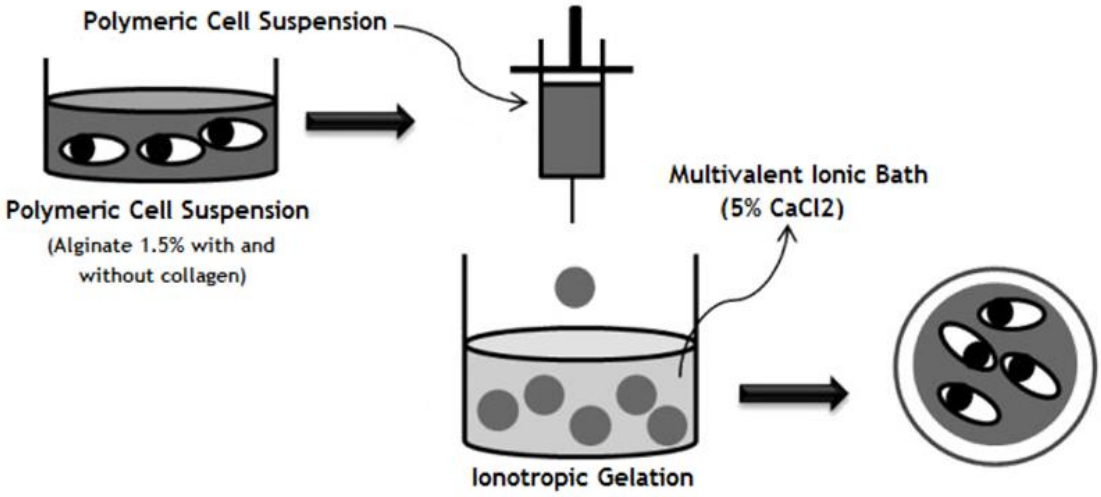


Figure 12: Representation of the process of human fibroblast cells encapsulation into alginate microparticles with and without collagen (adapted from [48]).

Microparticles without cells were also prepared to be used as control. Finally, the microparticles were placed in 96-well plate. Wells containing cells in the culture medium without materials were used as negative control (K^-). Ethanol (EtOH) 96% was added to the wells containing cells, and these wells were used as a positive control (K^+). The same number of cells was used in the controls and inside microparticles. Hereafter, cells and microparticles were kept in culture at 37°C in a 5% CO_2 humidified atmosphere, inside an incubator.

2.2.2. Optical Microscopy Analysis of the Microencapsulation System

Every day after cell encapsulation, the degradation of the alginate microparticles and cell release were monitored by using an Olympus CX41 inverted light microscope (Tokyo, Japan) equipped with an Olympus SP-500 UZ digital camera [48,68].

2.2.3. Scanning Electron Microscopy Analysis of the Microparticles

The microparticles morphology was analyzed through SEM at the 3rd, 10th and 17th day after cell encapsulation. Microparticles and their encapsulated fibroblast cells were fixed overnight with 2.5% glutaraldehyde in PBS at 4ºC. After that, samples were frozen at -80ºC. The microparticles with and without encapsulated cells were then dehydrated in 70, 80, 90 and 100% EtOH, during 5 min each. Next the samples were mounted on an aluminium board using a double-side adhesive tape and covered with gold using an Emitech K550 (London, England) sputter coater. Afterwards, the samples were analyzed using a Hitachi S-2700 (Tokyo, Japan) scanning electron microscope, working at an accelerating voltage of 20 kV and at various amplifications [23,38,48,68,97,98].

2.2.4. Fluorescence Labeling of Encapsulated Cells in Microparticles with Hoechst 33342

In order to prove that fibroblasts were encapsulated in microparticles, cells were stained with the nucleic acid staining dye, Hoechst 33342 (bisbenzimide trihydrochloride) and then confocal laser microscopy (CLSM) images were acquired [99]. This blue fluorescent dye has multiple applications in molecular biology and emits blue fluorescence when bound to dsDNA [100].

The labeling of the encapsulated cell nucleus was done by resuspending cells in DMEM-F12 with Hoechst 33342 molecular probe at a concentration of 500µg/mL, supplemented with 10% FBS without any antibiotic, in order to promote labeling. These suspensions were gently mixed and incubated during 30 min, in an incubator. Then, cells were encapsulated into sodium alginate with and without collagen by the method previously described in subsection 3.2.1.. The microparticles were placed in a glass bottom dishes coated with collagen and with 1.5mL of complete DMEM-F12. These culture dishes were placed at 37ºC, in 5% CO_2 humidified atmosphere. The culture medium was changed every 2-3 days [97,99].

Confocal and bright field images were obtained at the 3rd, 10th and 17th days after cell encapsulation, 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 appeared with a blue fluorescence. 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 of 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 [97,99].

2.2.5. Characterization of the Cytotoxic Profile of the Microparticles

After an incubation of 3 and 10 days, the fibroblasts viability inside of the two types of microparticles (n=5) was assessed through the reduction of a tetrazolium compound, MTS, into a water-soluble brown formazan product [101] (figure 13). 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.

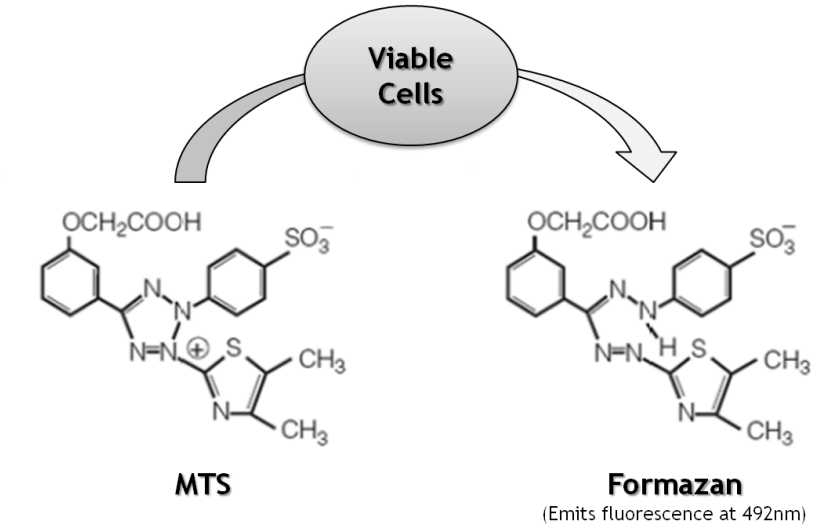


Figure 13: Reduction of the MTS into formazan by viable cells (adapted from [102]).

The medium was carefully removed in order to leave the microparticles in each well. Then, a mixture of 100µl of fresh culture medium and 20µl of MTS/phenazine methosulfate (PMS) reagent solution was added to each well. The cells were incubated for 4h at 37ºC, under a 5% CO_2 humidified atmosphere. The formazan absorbance was determined at 492nm using a microplate reader (Sanofi, Diagnostics Pauster). Wells containing only cells in the culture medium were used as K^- (negative control). EtOH 96% was added to wells containing cells to be used as a K^+ (positive control) [48,103].

2.2.6. Statistical Analysis of the Cytotoxic Profile Results

Statistical analysis of cell viability results was performed using one-way analysis of variance (ANOVA) with the Dunnet's post hoc test. Each result is the mean ± standard error of the mean of at least three independent experiments. A value of $p<0.05$ was considered statistically significant [23].

2.2.7. Synthesis of Deacetilated Chitosan

To address the influence of the presence of primary amine groups in the chitosan chain, the polymer was deacetilated and, subsequently, purified. Briefly, the chitosan flakes were dispersed in a NaOH solution. 500mg of (HMW) chitosan were mixed with 10mL of 1M NaOH. Then the mixture was heated at 50ºC, 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 40ºC overnight. The chitosan recovered was dissolved in 1M CH_3COOH solution [104].

2.2.8. Production of Chitosan Nanoparticles loaded with BSA

Chitosan nanoparticles were formulated using the ionotropic gelation technique. So, these chitosan nanoparticles were synthesized by intramolecular linkages between cationic polymers and negatively charged polyanions of TPP. Briefly, 0.75mg/mL of deacetilated (HMW) chitosan solution and a 0.5mg/mL solution of TPP were prepared. All the solutions were filtered with a 0.22µm filter to remove traces of solid particles. In order to promote encapsulation, BSA (2mg/mL) was added to the TPP solution prior to particle formation. Then, 1 mL of the BSA-TPP solution was added dropwise to 3mL of chitosan, under mild magnetic stirring conditions (300±50rpm), for 30 min, at room temperature. The formulated nanoparticles were washed and centrifuged (Hermle Z323K centrifuge) at 20000g, at 4ºC, for 30 min each one and sonicated for 15 min between each centrifugation [104,105].

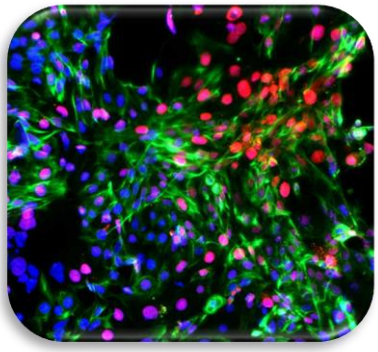
2.2.9. Determination of BSA Encapsulation Efficiency in Chitosan Nanoparticles

The encapsulation efficiency of the BSA inside the nanodevices was determined after isolation by centrifugation and recovery of the supernatant that contained the unbound protein. The amount of unencapsulated BSA was determined using the bicinchoninic acid (BCA) method. The absorbances were determined with an Ultraviolet-visible (UV-VIS) Spectrophotometer (UV-1700 PharmaSpec, Shimadzu) at 570nm. The encapsulation efficiency was determined through the following equation (1) [104]:

$$\text{Encapsulation Efficiency} = \frac{\text{Total Amount BSA} - \text{Free BSA}}{\text{Total Amount BSA}} \text{ X } 100\% \qquad \textbf{Eq. (1)}$$

2.2.10. Characterization of the Morphology of the Chitosan Nanoparticles

Particle morphology was analyzed by SEM using a Hitachi S-2700 (Tokyo, Japan) electron microscope working at 20kV and with different magnifications. Prior to image acquisition, the solutions with the nanoparticles were sonicated for 40 min. Then, samples were stained with 1% phosphotungstic acid and placed on a cover glass and vacuum dried at 37 °C overnight [104,106]. Afterwards the samples were mounted in microscope stubs and sputter coated with gold using an Emitech K550 sputter coater. All the particle samples were analyzed immediately after synthesis. Nanoparticle size was measured with the Roentgen SEM acquisition software version 1.3 [104].



Chapter III:

RESULTS and DISCUSSION

3.1. Morphology and Optical Properties of the Microparticles

Tissue engineering often involves the cell culture on biomaterials. Hydrogels are a class of biomaterials that have been used for cell encapsulation with therapeutic purposes [60]. They provide to encapsulated cells a 3D environment similar to that of the natural ECM of tissues, maintaining cells viable [60].

In the present study is described the development and optimization of a new microparticulated device to be used for wound healing treatment. To achieve this goal, human fibroblasts cells were encapsulated in two different types of microparticles: alginate and a mixture of alginate and collagen. To do so, fibroblasts cells were chosen, due to their potential for skin regeneration, since they synthesized proteins (e.g., collagen and fibronectin) of the ECM, cytokines and GFs (e.g., EGF and VEGF) that are essential for the wound healing mechanism. They are also the predominant type of cells in skin [11]. The microparticles, with and without cells, were produced using as gelling agent the $CaCl_2$. This method is the most commonly used to prepare hydrogels with an aqueous alginate solution that is combined with ionic cross-linking agents, such as divalent cations (i.e., Ca^{2+}) [71]. Macroscopic images of the microparticles are shown in figure 14A, where it can be depicted that the produced microparticles have a spherical and homogeneous shape. Figure 14B shows a microparticle at the microscopic scale, where it can be perceive the well-defined edge of the particle. Moreover, within the microcarrier, it is also possible to detect the presence of encapsulated cells with a spherical shape.

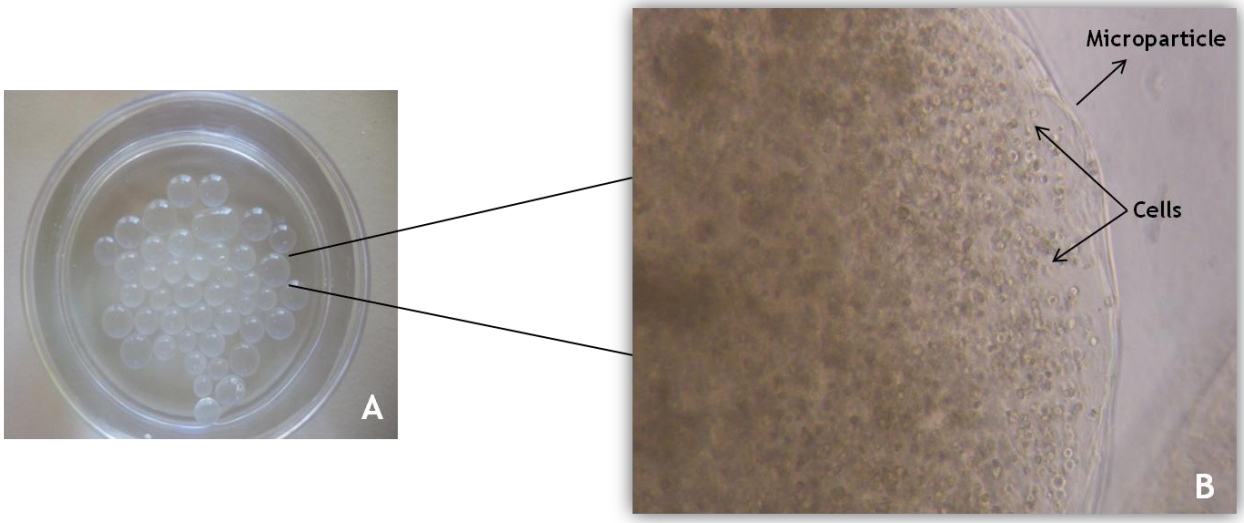


Figure 14: Macroscopic photograph of produced microparticles (A). Microscopic photograph of human fibroblast cells encapsulated in microparticles (B).

In this work, alginate microparticles with collagen were also produced. Collagen is known as a natural protein and a main component of ECM *in vivo* [107]. Its properties enables the growth of cells [108] and enhances the wound healing process, since it is able to fill defects, increase tensile strength of the wounds and promote the formation of new capillaries [109].

The morphology and shape of the microparticles without cells was characterized by SEM. The different microparticles produced, with and without collagen, presented a spherical shape and homogeneous morphology (see figure 15). Alginate microparticles presented a mean diameter of 1251µm, while the microcarriers produced with a mixture of alginate and collagen had a mean diameter of 1461µm. SEM results also demonstrated that the manufactured beads had a smooth and porous surface.

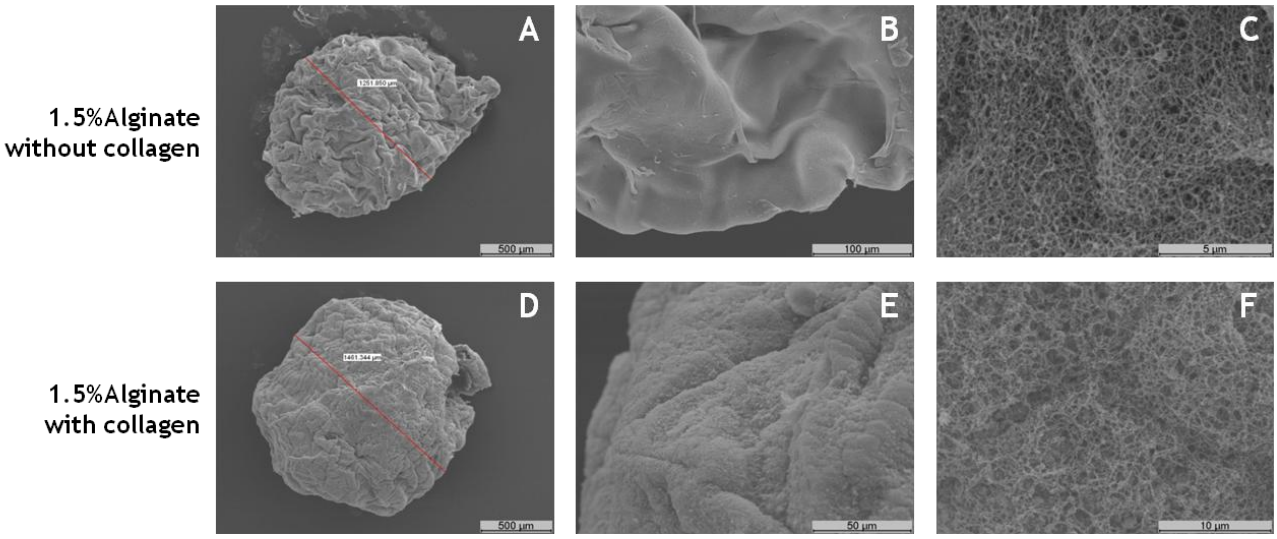


Figure 15: SEM images of the two types of microparticles without cells: 1.5% Alginate without collagen 50x (A), 350x (B), 8000x (C); 1.5% Alginate with collagen 50x (D), 700x (E), 4000x (F), after 3 days of being produced.

Both types of microparticles with cells encapsulated were also analyzed through SEM microscopy at the 3rd, 10th and 17th days after cells being encapsulated (figures 16, 17 and 18). The mean diameter of such microparticles was smaller than those without cells encapsulated. The mean diameters were 1115µm for alginate microparticles and 1142µm for the microparticles that were produced with alginate and collagen, respectively.

This difference between the microparticles with and without cells is probably due to the time of cross-linking of alginate with $CaCl_2$, which may have been slightly larger in the formation of microparticles with cells, since it promoted the synthesis of a tight alginate matrix thus diminishing the particle size.

The results presented in figures 16, 17 and 18 demonstrate that both types of microcarriers synthesized possess spherical and homogeneous morphologies. Interestingly, it is visible cells on the microparticle surface, which have a spherical shape.

From the 10th day onwards, it is possible to notice cells on the surface of the microparticles, which led us to the conclusion that from this starting point, cells started to be released from the microcarriers. When comparing the 17th day of both types of microparticles with encapsulated cells, it was possible to notice that more cells were observed outside the microparticles produced with alginate and collagen. Such achievement is not surprisingly since collagen is the most abundant structural component of the ECM, which provides support for cell adhesion and spreading [110].

It is also visible through the figures 16, 17 and 18 that the microparticles suffered some degradation over time, since they lose their spherical shape and size reduction. This fact allows cells to be released to the outside of the microparticles. The arrows that are shown in figures 16, 17 and 18 demonstrate the exact location of some encapsulated cells on the surface of the produced microparticles.

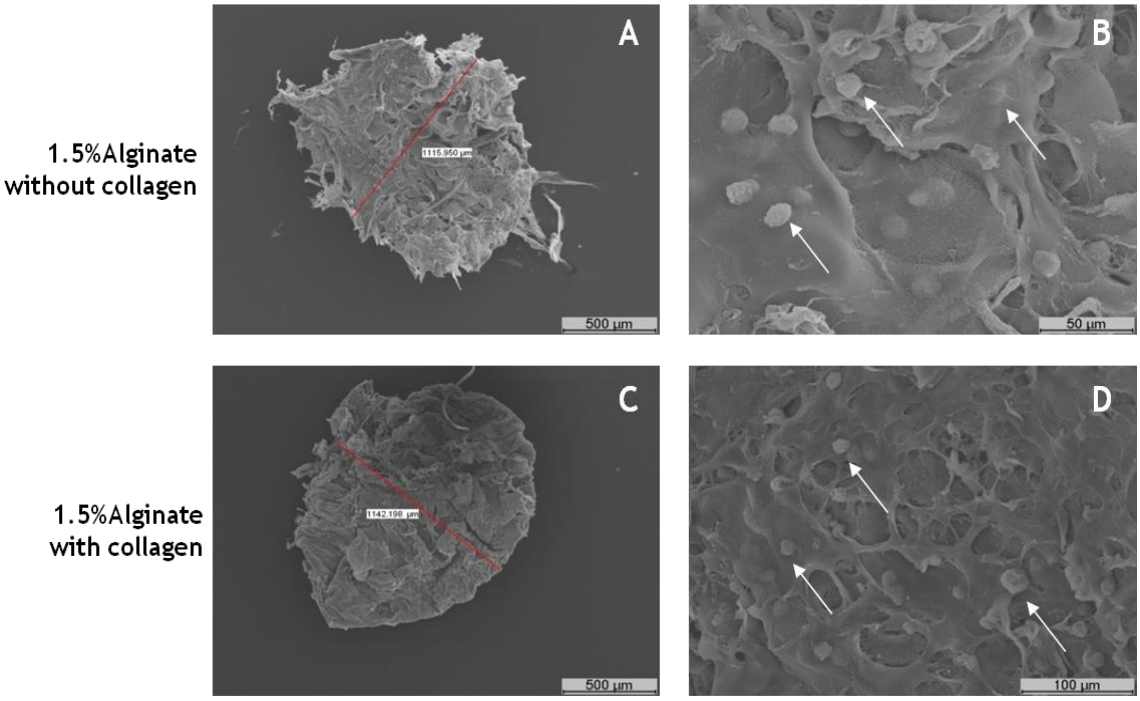


Figure 16: SEM images of human fibroblasts cells inside of the microparticles: 1.5% Alginate without collagen 50x (A), 500x (B); 1.5% Alginate with collagen 50x (C), 300x (D), after 3 days of being encapsulated.

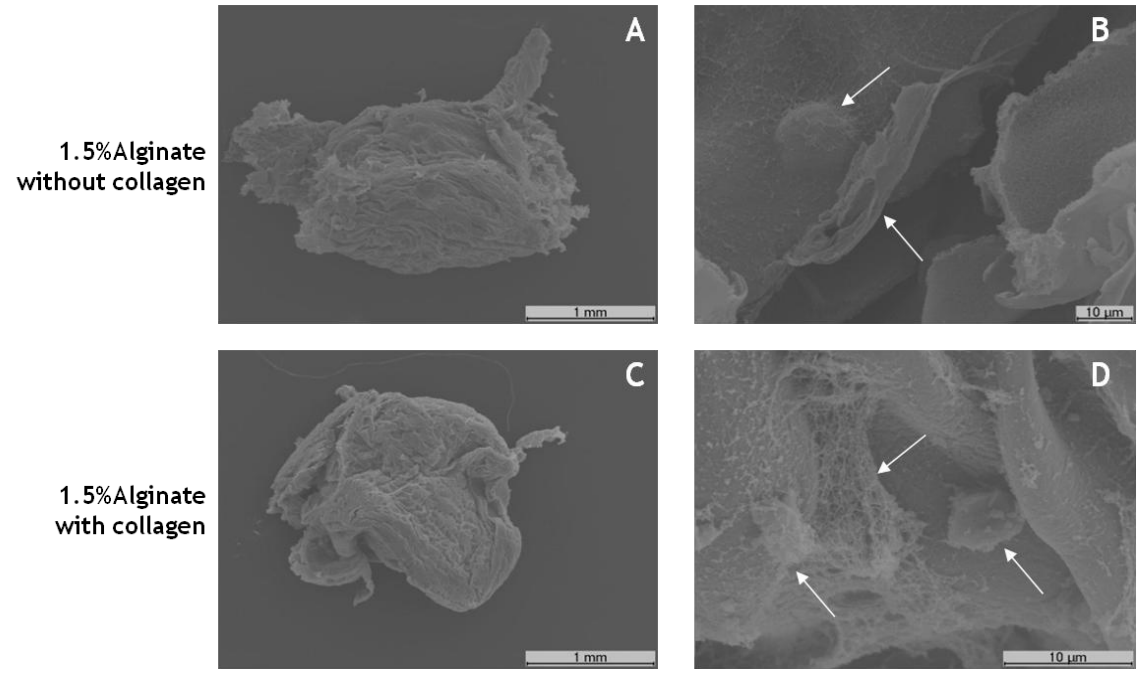


Figure 17: SEM images of human fibroblasts cells inside of the microparticles: 1.5% Alginate without collagen 35x (A), 1500x (B); 1.5% Alginate with collagen 35x (C), 3500x (D), after 10 days of being encapsulated.

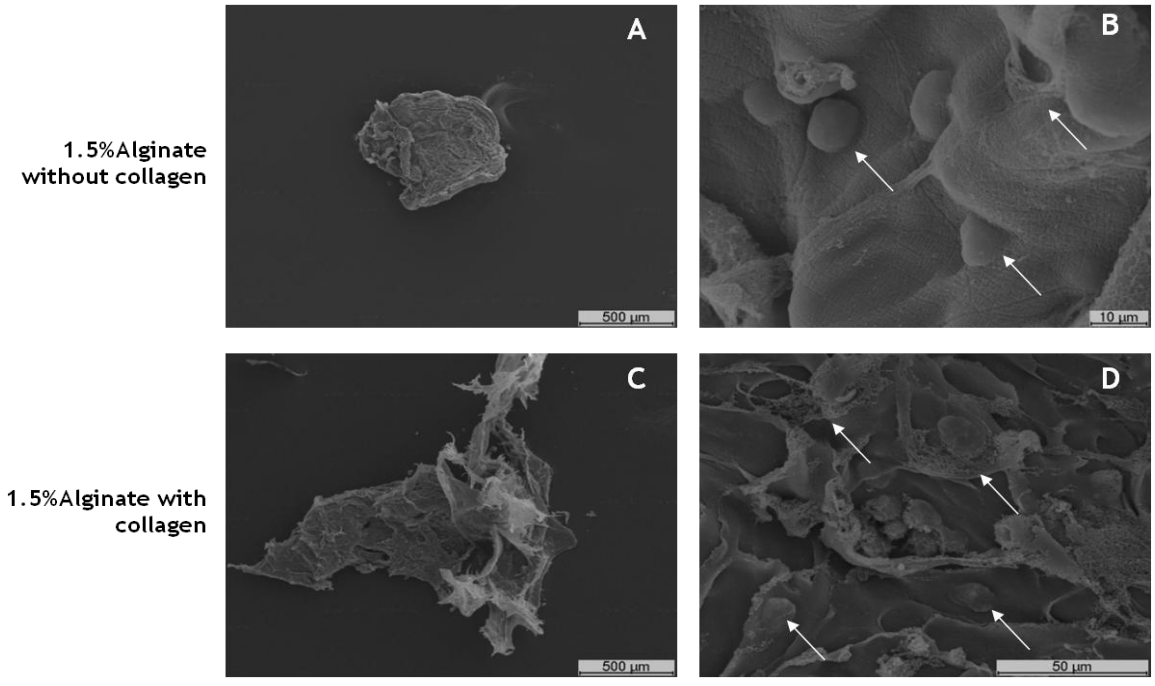


Figure 18: SEM images of human fibroblasts cells inside of the microparticles: 1.5% Alginate without collagen 50x (A), 1500x (B); 1.5% Alginate with collagen 50x (C), 800x (D), after 17 days of being encapsulated.

3.2. Microscopy Analysis of the Microencapsulation System

In this work, human fibroblasts cells were immobilized in alginate microparticles with and without collagen. Microparticles degradation and *in vitro* cell release were monitored every day after encapsulation, by using an inverted light microscope.

Through microscopic analysis it was possible to confirm that fibroblasts cells, when inside of the microparticles, had a round shape (figure 19A and B).

In the negative control (K^-), where cells were only seeded with DMEM-F12, cell adhesion and proliferation were observed. Live cells with their typical shape were visualized in figure 19C.

Still, in the positive control (K^+), neither cell adhesion nor proliferation was observed. Dead cells with their typical spherical shape are shown in figure 19D.

However, as the encapsulated cells and dead cells have the same spherical appearance it was necessary to assess the cytotoxic profile of the microparticles in order to prove the cell viability within the microcarriers. These results are discussed in subsection 3.4..

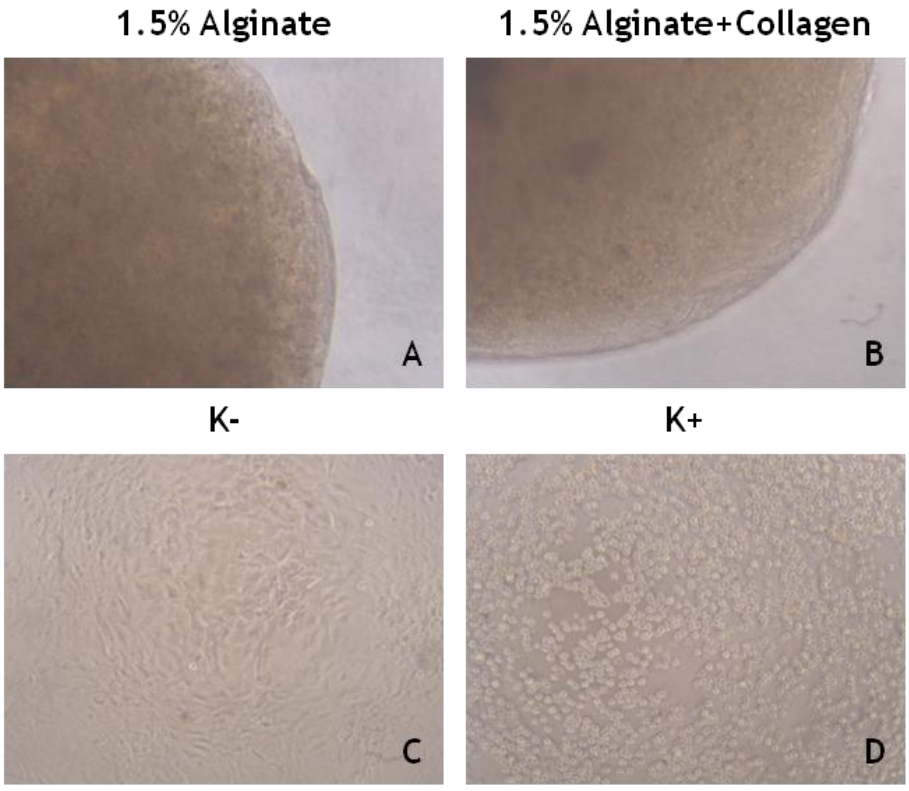


Figure 19: Microscopic photographs of human fibroblasts cells inside of the microparticles: 1.5% alginate without collagen (A); 1.5% alginate with collagen (B); negative control (K-) (C) and positive control (K+) (D), after 1 day of being encapsulated. Original magnification x100.

Moreover, by microscopy analysis, it was possible to verify that, 11 days after encapsulation, viable cells were seen outside the alginate microparticles without collagen. The cell release from alginate and collagen microparticles started only after 14 days of being encapsulated (figure 20A and B).

The observation of cell growth after their release from microparticles demonstrated that both microparticles were biocompatible and were suitable for cell encapsulation. These results showed that cells once inside of these 3D structures were able to receive nutrients and oxygen required for their survival. This can be easily explained by the fact that alginate polymer matrix is a porous structure, as already observed through SEM images (figure 15). The degradation of the alginate matrix during the incubation time also promoted the release of cells from the microcarriers.

It is important to refer that the cell release occurred approximately during 120 days, until the microparticles were completely degraded. The arrows shown in figure 20 indicate the location of some of the cells that have been released from the microparticles.

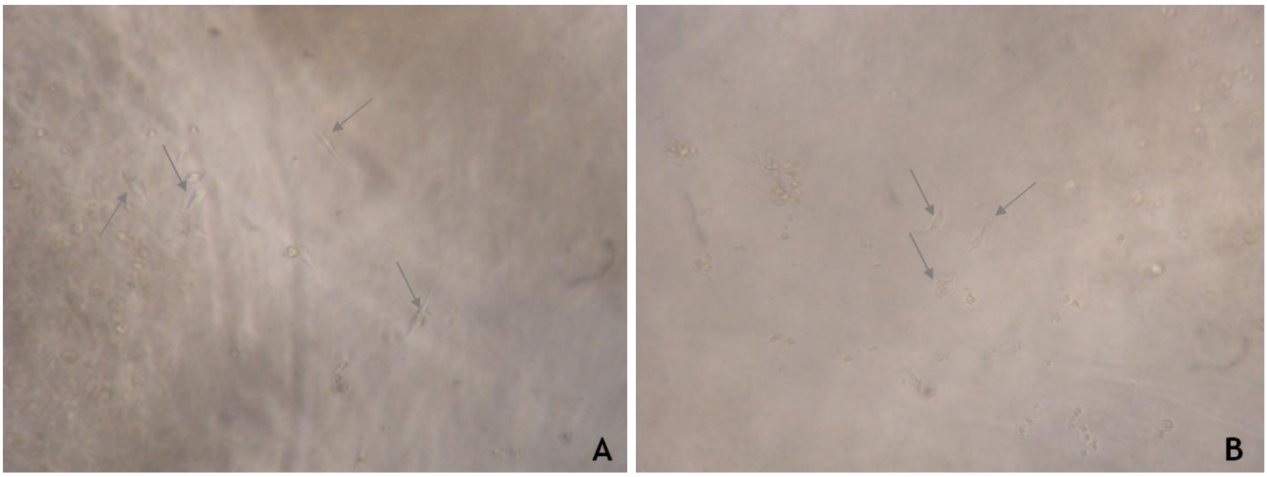


Figure 20: Microscopic photographs of human fibroblast cells inside and outside of the microparticles. Microparticles with 1.5% alginate (A) after 11 days of being encapsulated; microparticles with 1.5% sodium alginate and collagen (B), at 14th day post encapsulation.

3.3. Fluorescence Studies of Encapsulated Cells in Microparticles with Hoechst 33342

CLSM is an optical microscopy technique that has found tremendous utility in biology, biophysics, chemistry and materials science [111]. 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 [111]. The principle behind CLSM is to scan a focused laser beam inside a sample and collect the reflected or emitted light from the sample, while removing any light originating from 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 [111].

In order to show that cells were effectively encapsulated inside the alginate microparticles, CLSM images were obtained at 3rd, 10th and 17th days post encapsulation. Human fibroblasts cells were labeled with the blue nucleic acid staining dye, Hoechst 33342, for 30 min, before encapsulation. Next, the labeled cells were mixed with alginate solution with and without collagen. Both types of microparticles were produced by a gelation technique. After production of the microparticles, these were placed in glass bottom dishes with DMEM-F12 in an incubator.

Through the CLSM images (figure 21 and 22) obtained, it can be seen that cell encapsulation into the microparticles was achieved.

These images also give an idea of cells location inside of the microparticles. Through images analysis, it can be seen that in both types of alginate microparticles the cells were dispersed along the carriers. However, it was also noticed that there are more cells in microparticles periphery. Such feature can be explained by the greater diffusion of oxygen and nutrients essential for cell survival, which occurs better at the edges of the particles. Some cells clusters could also be seen.

The results obtained showed that our method was effective for human fibroblasts cells encapsulation into microcarriers.

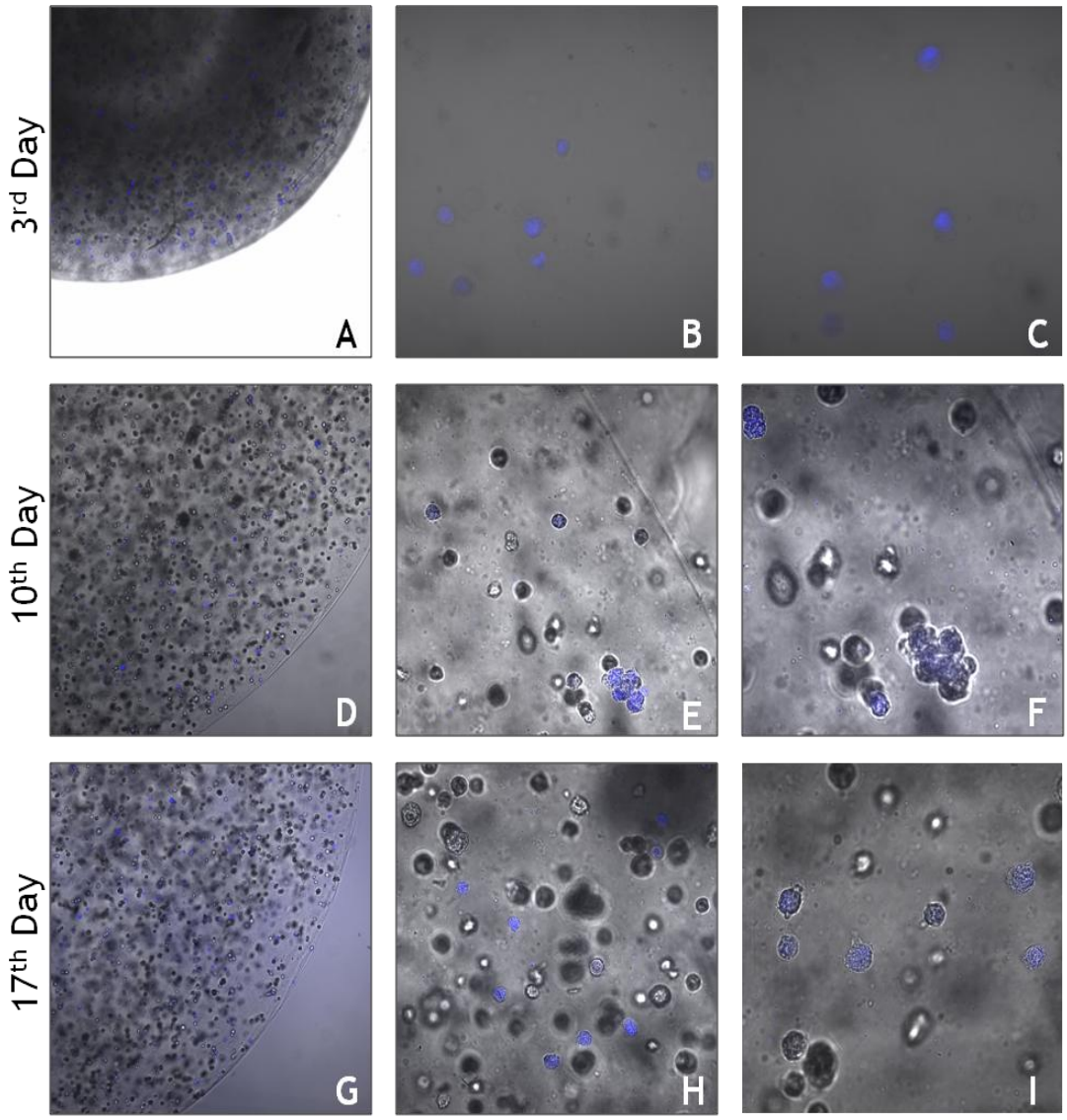


Figure 21: CLSM images of human fibroblasts cells inside the alginate microparticles after 3, 10 and 17 days of being encapsulated. 100x (A), 400x (B), 630x (C); 100x (D), 400x (E), 630x (F), 100x (G), 400x (H), 630x (I).

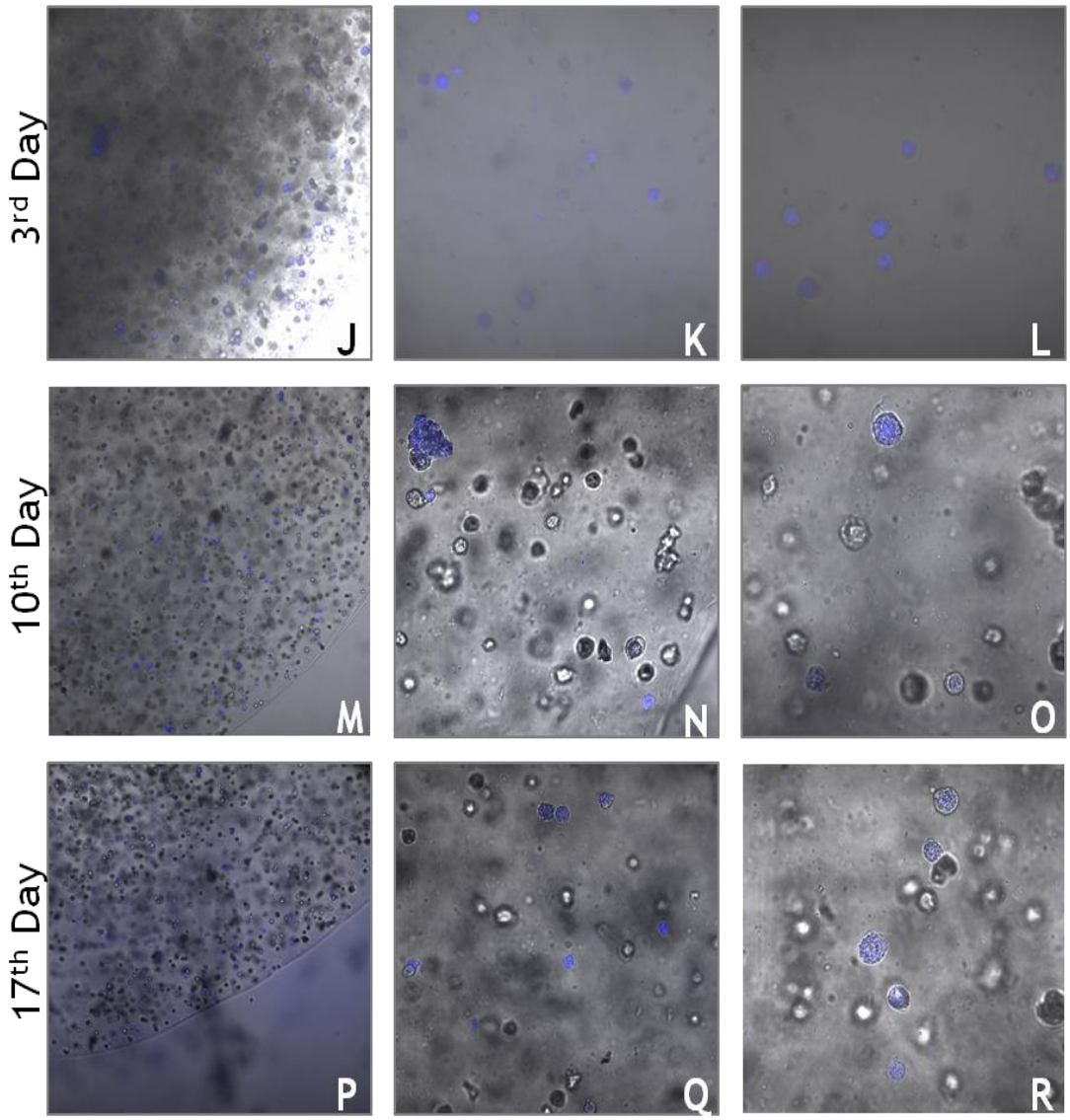


Figure 22: CLSM images of human fibroblasts cells inside the alginate with collagen microparticles after 3, 10 and 17 days of being encapsulated. 100x (J), 400x (K), 630x (L); 100x (M), 400x (N), 630x (O), 100x (P), 400x (Q), 630x (R).

3.4. Characterization of the Cytotoxic Profile of the Microparticles

The cytocompatibility of alginate microparticles with and without collagen was characterized through a MTS assay. As already mentioned, human fibroblasts cells were encapsulated in the two types of microparticles. Microparticles without cells were also prepared to be used as a control. Afterwards, the different microcarriers were placed in 96-well plate with DMEM-F12. The viability of the encapsulated cells was assessed over time (at the 3rd and 10th days after being encapsulated). Cell viability was quantitatively measured using this MTS non-radioactive assay. The MTS is a tetrazolium compound which is reduced by living cells to yield a water-soluble formazan product, which is determined at 492nm [103].

The MTS assay results (figure 23) showed that cells viability was high for the negative control (K^-), where cells were only seeded in the presence of DMEM-F12.

Through the analyses of figure 23, it is also possible to state that fibroblasts cells encapsulated in microparticles with and without collagen had metabolic activities similar to that of K^-, since the same number of cells were encapsulated in the microcarriers. However, at day 10, it can be seen that there was a slight increase in cell viability in the microparticles with collagen when compared to the microparticles without this protein. Such, result can be explained based on the use of collagen that, as already mentioned, is a component of ECM and thereby promotes cellular adhesion and, subsequently, cellular proliferation [107]. As expected the positive control (K^+) showed almost no viable cells where the cell viability is very low, as it can be confirmed in figure 23.

The MTS assay also showed a significant difference between the positive control ($p<0.05$), the negative control and cells encapsulated in microparticles. However, no significant difference was observed for cells being encapsulated for 3 or 10 days in the two carriers.

So, these facts demonstrated that these compounds used to produce the microencapsulation systems did not affect cell viability and can be used in a near future for skin regeneration. These results also allow to affirm that, once the cells are viable within both types of microparticles, they start to produce all the bioactive molecules that are essential for the wound healing mechanism after being encapsulated, even just they began to release of the microparticles of alginate and alginate with collagen after 11 and 14 days post encapsulation, respectively.

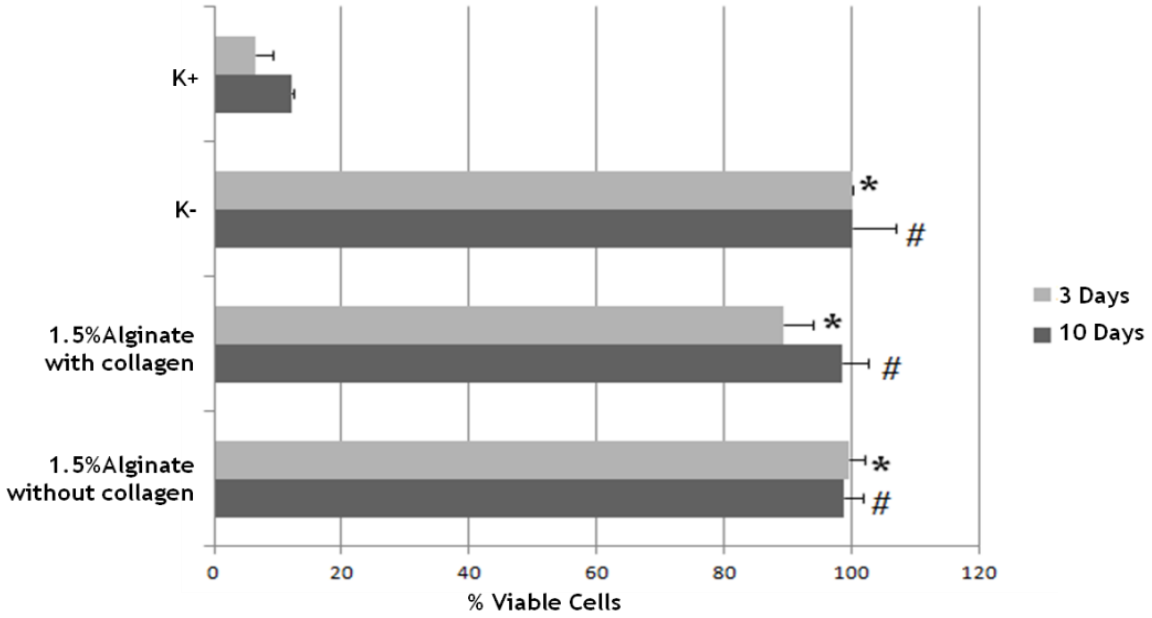


Figure 23: Evaluation of cell viability by a MTS assay after 3 and 10 days of being encapsulated into microparticles. Alginate microparticles (1.5% Alginate without collagen); alginate and collagen microparticles (1.5% Alginate with collagen); negative control (K-); positive control (K+). Each result is the mean ± standard error of the mean of at least three independent experiments. Statistical analysis was performed using one-way ANOVA with Dunnet's post hoc test (*$p < 0.05$; # $p < 0.05$).

3.5. Characterization of BSA loaded Chitosan Nanoparticles

Chitosan nanoparticles were prepared by ionic gelation method using TPP. The formation of nanoparticles is based on an ionic interaction between positively charged chitosan and negatively charged TPP [53,81,97]. The ionic cross-linking process is attractive in the preparation of protein delivery devices due to its simple and mild procedure, without the need of apply harmful organic solvents, which decreases protein denaturation and degradation [112].

The formation of chitosan–TPP nanoparticles occurred spontaneously upon the addition of the TPP ions to the chitosan solution. BSA was chosen as a model protein to be loaded in nanoparticles [78]. Its molecular weight is 66 kDa and has an isoelectric point (pI) of 4.7 in water (at 25 °C). It is widely used for drug delivery studies because of its abundance, low cost, easily purified and its acceptance by the pharmaceutical industry [78].

The nanoparticles herein studied were produced with deacetylated chitosan. The DD can be measured by determine the first derivative of an UV-spectroscopy spectra, as described by Gaspar *et al*. [104]. Chitosan DD obtained through this method has a DD above 98% [104]. This means that almost all of the primary amine groups of the chitosan polymer chain were positively charged and therefore ready to react not only with TPP, but also with BSA, influencing the final nanoparticle size.

The morphological characteristics of BSA-incorporated nanoparticles were examined using SEM and it revealed that the nanoparticles obtained have a size range between 76nm and 156nm. Nanoparticulated systems produced showed randomly shaped with a very well defined spherical morphology, as depicted in figure 24.

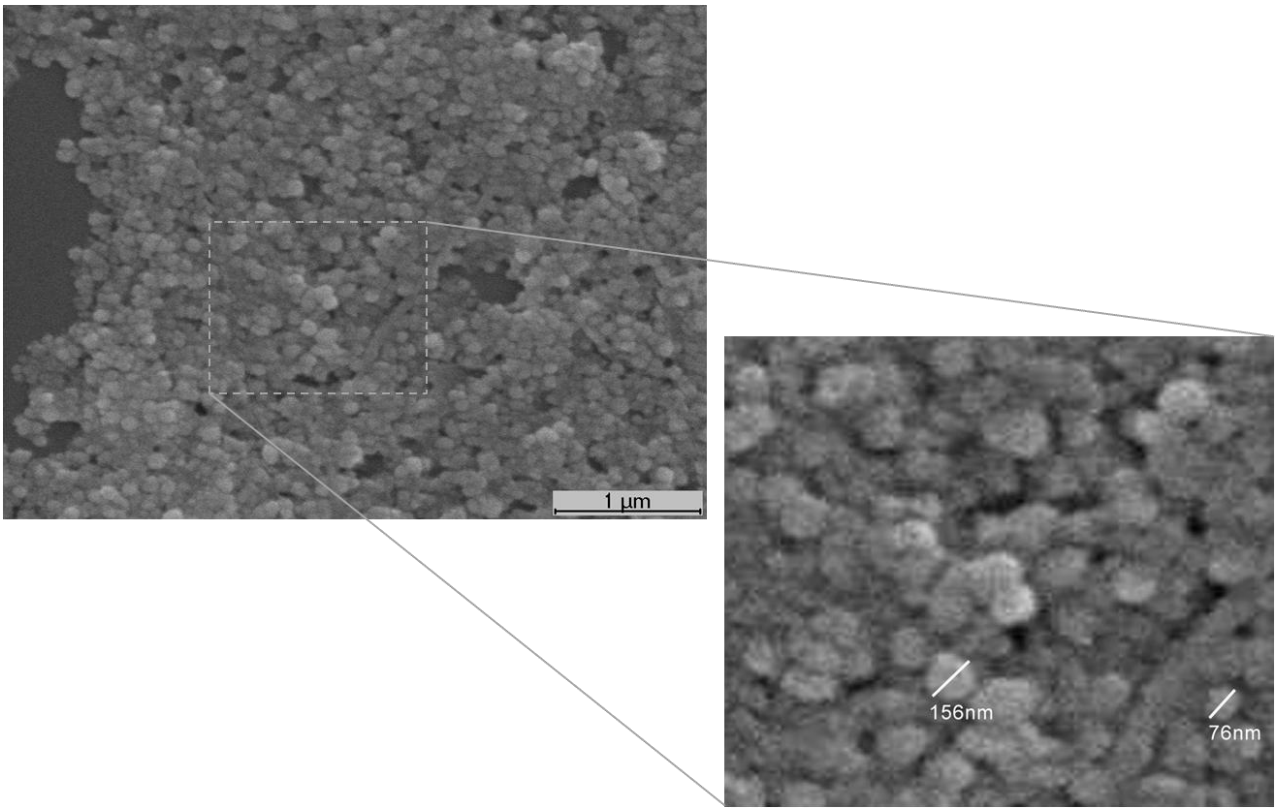


Figure 24: Characterization of the morphology of the nanoparticles produced with HMW deacetilated chitosan by SEM.

3.6. Encapsulation Efficiency of BSA in Chitosan Nanoparticles

The protein entrapment efficiency, which corresponds to the amount of drug that can be incorporated in the nanoparticles either inside the particle or adsorbed at the surface, was determined indirectly by measuring the concentration of the free protein in the aqueous phase of the nanoparticle dispersion [70].

In the present work, for the encapsulation process, BSA was included in the TPP solution prior to chitosan nanoparticle formation.

BSA entrapment efficiency was calculated by the BCA method. This BCA protocol allows for an accurate, rapid and economical estimation of the peptide concentration within a sample. It depends on the conversion of Cu^{2+} to Cu^{+} by proteins under alkaline condition allowing this ion detection trough BCA assay. This, results in the development of an intense purple color with an absorbance maximum at 562nm. Since the production of Cu^{+} in this assay is a function of protein concentration and incubation time, the protein content of unknown samples may be determined spectrophotometrically by comparison with known protein standards [113,114], which are given by the calibration line and is represented in figure 25. This calibration curve depicts the relationship between known concentrations of BSA and their respective absorbances. From the trend line equation, it was possible to extrapolate the encapsulation efficiency of BSA, which was approximately 79%.

These results demonstrated that these nanoparticles are suitable for protein entrapment, since they have a similar protein encapsulation efficiency when compared with other research studies [105,115].

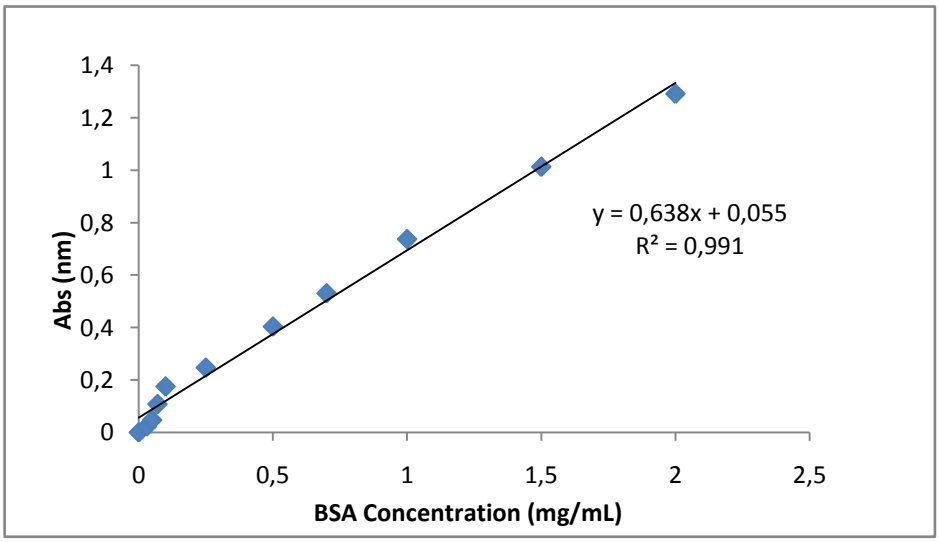
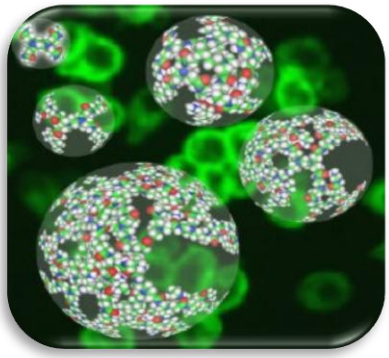


Figure 25: Calibration line and its respective equation used to calculate the encapsulation efficiency of BSA in chitosan nanoparticles.



Chapter IV:

CONCLUSIONS and FUTURE PERSPECTIVES

4. Conclusions and Future Perspectives

Skin lesions are traumatic events that remain as a leading cause of morbidity and mortality of injured patients. After skin damage, wound healing is initiated. Such issue is a complex biological process that includes different events. Despite of its complexity, skin regeneration is often defective. Over the past few years, many skin substitutes have been developed. However, none of the skin substitutes available or even under development are able to fully replace the properties of the native skin. Skin engineering requires a scaffold that provides the cells an adequate microenvironment that promotes cell adhesion and proliferation, and allows the diffusion of bioactive molecules. With the advances in tissue engineering, the entrapment of cells inside hydrogels was become possible. Therefore, cell encapsulation arises as a new strategy to an *in situ* cell delivery directly into the injury site. This methodology allows cell encapsulation into microparticles and the continuous delivery of secreted proteins by cells, to treat different pathological conditions. Spherical microparticles offer optimal surface-to-volume ratio for protein and nutrient diffusion, and thus, contribute for cell viability. This technology also allows the cell to survive without the deleterious effects of immunosuppressant drugs.

In this work, two different kinds of microparticles were produced in order to encapsulate human fibroblasts cells with the ultimate goal of being used to improve the wound healing mechanism. The two types of microparticles were produced one with sodium alginate and other with sodium alginate and collagen. Collagen was added to microparticles in order to improve cell viability, since it is the most abundant structural protein of the ECM. Thus, the combination of a polysaccharidic polymer (in this case alginate) with a bioactive peptide (collagen) is fundamental for inducing skin regeneration and improving the wound healing process and it also allows adhesion between cells.

The microscopy and CLSM analysis showed that cells were encapsulated inside the two types of microparticles. Through microscopic analysis, it was visualized that the cells began to be released from the microparticles after only few days of encapsulation. In the case of alginate microparticles, the cells began to release the microparticles after 11 days of being encapsulated. While in the microparticles of alginate and collagen was observed cells outside the microcarriers after 14 days of being encapsulated. Thus, it can be stated that the polymeric matrix is semi-permeable. This fact also allows the diffusion of nutrients and oxygen necessary for cell survival and of all bioactive molecules secreted by encapsulated cells to the outside of them, which are needed for tissue regeneration. The morphology of the microparticles was also characterized by SEM analysis. Cell viability inside microparticles was assessed by a MTS assay. This study confirmed that cells were viable within the microparticles. Thereby, we concluded that, based on our *in vitro* studies, the developed microparticles can be used as a cellular microencapsulation system.

In the second part of the study, an appropriate chitosan nano-system was also synthesized to extend the period among which the bioactive molecules were release from the microcarriers, therefore overcoming some handicaps correlated with the short half-life and rapid diffusion of these macromolecules. Due to its cationic nature and controlled degradation rate, chitosan based nanodevices have occupied a reasonable place in drug delivery. The chitosan nanoparticles were successfully produced by ionotropic gelation with TPP. Such nanodevices were morphologically characterized by SEM, showing mean size of 150nm. We also investigated the protein entrapment efficiency by using BSA as a model protein. The encapsulation efficiency of BSA, calculated by a BCA method was 79%. Based on this result, it was possible to conclude that this delivery system appears to be a good candidate to be used for controlled release of GF in skin regeneration.

In a near future, the developed chitosan nanoparticles will be encapsulated inside of the alginate microparticulated systems herein produced. The applicability of these systems will be further characterized through *in vitro* and *in vivo* assays. When introducing chitosan nanoparticles into alginate microparticles, the quantity of drug to be released from such nanodevices will release in a controlled way and for a longer period of time. The carriers herein developed can also be used for the delivery of others bioactive substances, such as GFs and nucleic acids.

Chapter V:

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