FACULDADE DE ENGENHARIA DA UNIVERSIDADE DO PORTO

Development of wound dressings based on a pectin and chitosan interpenetrating polymer network

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Resumo

A pele, sendo o maior órgão do corpo humano, cobre toda a sua superfície externa, e é uma estrutura organizada e complexa composta por três camadas sequenciais e múltiplos apêndices. A pele é um órgão extremamente importante, desempenhando inúmeras funções, tais como proteção, perceção e regulação, mas também é muito vulnerável a sofrer danos. As feridas são lesões ou perturbações à normal estrutura anatómica, comprometendo as funções do tecido. Apesar de estar em constante renovação, a capacidade de auto-cura da pele não é ilimitada. Perder a barreira que a pele fornece pode acarretar consequências severas aos pacientes, e o número de pessoas a sofrer de feridas da pele tem vindo a aumentar. Pensos de feridas são extremamente necessários em qualquer tratamento de feridas da pele, de modo a protegê-las de infeções e desidratação. Esses pensos têm de ser biocompatíveis, apresentar propriedades físicas e mecânicas adequadas, ser fáceis de manusear, flexíveis, robustos, aderirem ao local da ferida, apresentar boas propriedades de dilatação para remover excessos de exsudados, ter uma boa taxa de transmissão de vapor de água, permitir trocas de gases e providenciar um ambiente quente e húmido para auxiliar no processo de cura. Este projeto teve como objetivo a produção de esponjas compostas por dois polímeros naturais (pectina e quitosano) através da reticulação física entre eles usando $CaCl_2$ e NaOH, formando uma rede interpenetrante de polímeros. O objetivo é que estas esponjas funcionem como pensos. Primeiramente foi levado a cabo um processo de otimização, de modo a selecionar alguns parâmetros. De seguida, as amostras produzidas foram caraterizadas relativamente às suas propriedades mecânicas, de absorção de água, de perda de água, de transmissão de vapor e de degradação, de modo a avaliar o seu potencial para ensaios biológicos subsequentes. Estas propriedades foram avaliadas em detalhe, sendo que as amostras produzidas apresentaram propriedades adequadas para serem usadas como pensos, comparando com valores da pele e com outros pensos já produzidos e descritos na literatura. As diferentes formulações produzidas apresentaram propriedades similares entre si, mas cada uma pode ser usada para uma dada aplicação desejada com base nas suas diferenças.

Abstract

Skin, the largest organ of the human body which covers its entire external surface, is an organized and complex structure, composed of three sequential layers and several appendages. Skin is an extremely important organ, performing several functions, such as protection, perception and regulation, but is also very vulnerable to damages. Wounds are injuries or disruptions of the normal anatomical structure, compromising tissue functions. Although the skin is under constant renovation, its self-healing ability is not unlimited. Losing the skin barrier can bring severe consequences to patients, and the number of people suffering from skin wounds is growing. Wound dressings are extensively needed in any type of wound treatment, in order to protect it from infection and dehydration. They have to be biocompatible, present adequate physical and mechanical properties, be easy to handle, be flexible, be robust, adhere to the wound bed, hold an excellent swelling ability to remove the excess of exudates, have an optimal water vapor transmission rate, allow gaseous exchanges and provide a moist and warm environment to the wound bed and promote wound healing. This project was aimed to develop sponges based on natural polymers (pectin and chitosan) by the physical crosslinking between them using $CaCl_2$ and NaOH, forming an interpenetrating polymer network. These sponges are aimed to be used as wound dressings. Firstly, a qualitative optimization was carried out, in order to select some parameters. Afterwards, the produced dressings were characterized regarding mechanical properties, swelling, water loss, vapor transmission and degradation, in order to evaluate their potential for subsequent biological evaluation. These properties were evaluated in detail, being that the produced samples presented adequate properties to perform as wound dressings, comparing with values of skin and with other dressings already produced and described in the literature. The different produced formulations presented very similar properties between them, but can be used according to different applications based on their differences.

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This work would not be possible without the presence and help of the people that contributed to all my academic life in the past five years.

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Hugo Miguel Nunes Fernandes

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Abbreviations and Symbols

Aw	Absorption of water
CAD	Computed-aided design
CAM	Computer-aided manufacturing
CT	Computed tomography
ddH2O	Deionized water
DE	Degree of esterification
DMEM	Eagle's minimal essential medium
E	Youngs' modulus
EC	Endothelial cell
ECM	Extracellular matrix
EWC	Equilibrium water content
EWL	Evaporation water loss
e.g.	*Exempli gratia* - for example
FGF-2	Fibroblast growth factor 2
FTIR	Fourier transform infrared
GA	Glutaraldehyde
GAGs	glycosaminoglycans
hMSC	Human Mesenchymal stem cell
i.e.	*Id est* - that is
IPN	Interpenetrating Polymer Network
LAB	Laser-assisted bioprinting
MRI	Magnetic resonance imaging
MPa	mega pascal
p	p value
PBS	Phosphate-buffered saline
PCL	Polycaprolactone
PEC	Polyelectrolyte complex
PECMA	Pectin methacrylate
PEG	Polyethyleneglycol
PGA	Polyglycolic acid
PLA	Polylactic acid
PLGA	Poly(lactic-co-glycolic acid)
RGD	Arginine (Arg)-glycin (Gly)- aspartic acid (Asp)
RM	Regenerative medicine
SD	standard deviation
SEM	Scanning electron microscopy
SMCs	Smooth muscle cells

TE	Tissue engineering
TERM	Tissue engineering and regenerative medicine
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
v/v	volume/volume
WVTR	Water vapor transmission rate
w/v	weight/volume
XRD	X-ray diffraction
2D	Two-dimensional
3D	Three-dimensional
ºC	Celsius degrees
μs	Microseconds
μm	Micrometers

Chapter 1

Introduction

This Chapter contains a general overview of skin anatomy, physiology, constituents and functions, as well as the mechanisms underlying the wound healing process, in order to understand the object of this dissertation project. The need of treatments and products for skin care, namely wound dressings, is also addressed.

Skin is the largest organ of the human body, accounting for about 15% of the total adult body weight [9]. Moreover, it covers the body's entire outer surface. Therefore, the skin proves to be an extremely important structure for the human body.

1.1 Skin Functions

The main function of the skin is to protect the whole body, acting as a barrier against the external environmental dangers, be them physical, chemical, or biological [10]. It protects against UV radiation, chemical exposure, diffusion of molecules and penetration of foreign pathogens, having also an important immunological function [11]. Being impermeable, it prevents water loss for the surrounding environment, maintaining the hydration of the organism. The skin has adequate mechanical properties, such as resistance, flexibility and elasticity, to act as a barrier and at the same time allowing free movement [12].

The skin has also an important role in the sensory function, because it is densely innervated with sensory nerve fibers that sense touch, temperature, and pain [13, 14].

The skin is also important on the regulation of the body temperature, by secreting sweat through sweat glands, which is then evaporated, and by regulating the blood flow in the more superficial blood capillaries [15].

When exposed to sunlight, skin produces vitamin D, which is a crucial molecule for calcium absorption, contributing to the health of bones [16, 17].

Finally, skin also has an exocrine function, by secreting sweat and sebum, and it has metabolic functions, as well as aesthetics functions.

1.2 Skin Structure and Constituents

The skin is an organized and multilayered organ, composed by 3 successive layers - epidermis, dermis, and hypodermis (**Figure 1.1**) - each layer having distinct biomechanical properties, functions, components and cell populations (**Table 1.1**). It also contains appendages, which will be discussed.

1.2.1 Epidermis

Epidermis is the most superficial skin layer, composed of keratinized stratified squamous epithelium [9]. This layer protects the underlying tissue.

The main cellular constituents of the epidermis are keratinocytes [18], basal cells [19], melanocytes [20], Merkel cells [21] and Langerhans cells [22]. Keratinocytes are the epidermis' main cell population (95-97% [23]). Although, the epidermis is an avascular layer, but it is under constant renewal due to the presence of basal cells, which are stem-like cells, situated in the basal layer [19].

The epidermis is subdivided into 4 or 5 layers [11, 24], containing keratinocytes in distinct stages of keratinization, differentiation, and proliferation. From surface to depth, the layers are:

- ***Stractum corneum***: It is the outermost layer, constituted by keratin and horny cells (or corneocytes) [25] (dead cells). It protects the underlying layers, preventing invasion by foreign substances and water loss. This layer displays thickness changes as a function of the body location;
- ***Stractum lucidum***: It is a thin layer consisting of dead cells containing dispersed keratoahylain [26]. It is not always present;
- ***Stractum granulosum***: It is composed of flattened cells retaining keratohyaline granules in their cytoplasm;
- ***Stractum spinosum***: It is composed of keratin [27] filaments and a variety of cells with different structures and shapes;
- ***Stractum basale* or *Stractum germinativum* or basal layer**: It is separated from dermis by the basement membrane or basal lamina [28], which is a porous structure, allowing the exchange of cells and fluids. This layer is the first location of epidermis mitotically active cells (as will be discussed on **Section 1.3**).

1.2.2 Dermis

The middle layer of the skin is the dermis, which is a dense connective fibrous tissue. Together, the epidermis and the dermis form the cutis, which usually presents a thickness of between 1.5 and 2.5 mm [29], but this value can vary considerably according to the location on the body.

The dermis is composed of an intricate network of ECM molecules surrounding tissue-resident cells (e.g., fibroblasts, macrophages) and functional structures such as blood vessels. This ECM is fundamentally made up of interconnected collagen fibrils (18–30% of the volume and 70-80% of the fat-free dry mass of dermis [30]), elastic fibers (elastin), proteoglycans, and glycoproteins Collagen [31] is a fibrillar structural protein responsible for the skin tensile strength. On the other hand, elastic fibers [32] provide elasticity to the skin.

The predominant cells in dermis are the fibroblasts [33], responsible for ECM components production and maintenance. They produce collagen, growth factors, glycosaminoglycans (GAGs) and fibronectin, and thus play a key role in wound healing. Other types of cells can also be found in the dermis, namely: smooth muscle cells (SMCs) and endothelial cells (ECs) lining the blood vessels, as well as macrophages and mast cells, which are responsible for surveillance and immune and inflammatory responses [34].

The dermis provides mechanical and structural support to the epidermis, and provides flexibility and strength to the skin. Dermis is a highly dynamic layer and also contains blood and lymphatic vessels, the skin appendages (explored in **Section 1.2.4**) and sensory receptors [35].

The dermis consists of 2 connective tissue layers merging together without a clear demarcation [24]:

- **Papillary layer**: It is the outer layer, contacting directly with the epidermis. It is composed of lose connective tissue and it presents higher cellular density;
- **Reticular layer**: It is the deepest layer of the dermis. It consists of dense connective tissue with collagen fibers. It is thicker and less cellular than the papillary layer.

1.2.3 Hypodermis

Also called subcutaneous tissue, hypodermis is the deepest layer of the skin. It is a fatty layer mainly composed of adipose cells (adipocytes) [36], along with some blood vessels, sensory neurons, connective tissue, and skin appendages [24]. The subcutaneous tissue is a source of energy for the body and it also acts as an heat insulator.

1.2.4 Skin appendages

- **Sweat glands** [37]: Tubular structures that regulate body temperature through evaporation of released sweat;
- **Hair follicles** [38]: Important source to grow keratinocytes in re-epithelialization. They can keep the body warm, although this is not particularly relevant in humans;
- **Oil or sebaceous glands** [39]: Secrete sebum, which is an oily matter that lubricates and moistures hair and the epidermis.

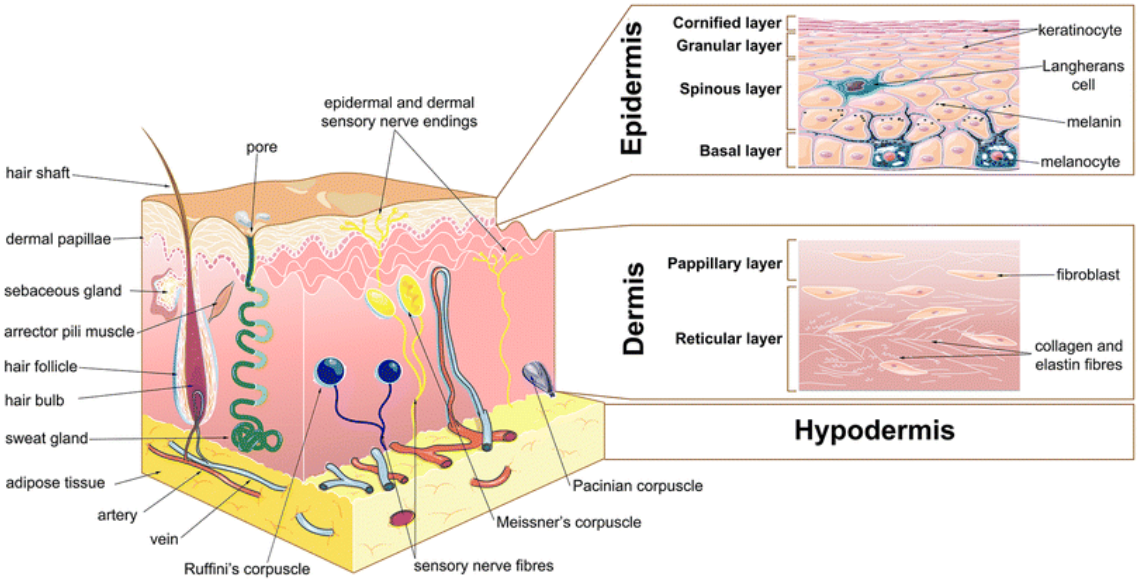

Figure 1.1: Schematic representation of the multi-layered structure of human skin. The 3 skin layers, epidermis, dermis, and hypodermis, and their constituents. Image from Pereira *et al.* (2017) [1].

Table 1.1: Skin cells: location and main functions

Skin layer	Cell Type	Main functions
Epidermis	Keratinocytes	Synthesis of keratin (protein which provides strength and protection to the skin)
	Melanocytes	Production of melanin (pigment which confers coloration to the skin, protecting cells against UV radiation, which production is stimulated by the exposure to UV light itself)
	Langerhans cells	Dendritic cells of the immune system, derived from the bone marrow. Involved in some T-cell responses
	Merkel cells	Tactile epithelial cells; mechanoreceptors essential for touch sensation. Found in the lips, digits and outer root coating of the hair follicle
	Basal cells	Located at the basal layer, they are the epidermal stem cells (proliferate and self-renew, ensuring tissue renewal)
Dermis	Fibroblasts	Production of ECM components
	Macrophages and mast cells	Responsible for surveillance and for immune and inflammatory responses
Hypodermis	Adipocytes	Energy storage and secretion of molecules involved on metabolism

1.3 Epidermis Proliferation and Differentiation

The skin is under constant renovation and remodeling. Therefore, in order to maintain a relatively constant cell quantity, there must exist a strict balance between cell proliferation, differentiation and apoptosis [40], i.e. programmed cell death [41].

Epidermal morphogenesis and differentiation are regulated by the underlying dermis. The basal cells (skin stem cells [19]) of the epidermis undergo proliferation cycles, providing cells for the renewal of the outer epidermis [41]. These mitotically active cells are located on the basal layer and give rise to cells of the outer epidermis layers.

Keratinocytes first pass through a synthetic phase and then a degradative phase, turning into corneocytes. This process is called keratinization [42], being the differentiation process that occurs as the cells migrate from the basal layer to the skin surface. The process of maturation ultimately results in cell death, and is known as terminal differentiation [9].

1.4 The Wound Healing Process

A wound can be defined as a disruption of the normal anatomical structure that compromises tissue function, and it can be in different extents [43]. Depending on the underlying cause, wounds can be classified into 2 categories:

- **Acute wound** [44]: It is a skin injury that occurs suddenly rather than over time. It heals in a predictable way according to the normal wound healing process. It is mostly caused by mechanical or chemical injuries, burns, or surgical incisions.
- **Chronic wound** [45]: These are wounds that do not heal in an orderly set of stages, exhibiting a slow healing rate, and often involve significant tissue lost. This type of wound often remains in the inflammatory stage for too long, and may never heal, causing pain and aesthetics concerns to the patients. Major causes of chronic wounds are ulcer caused by vascular insufficiency, diabetes mellitus and local-pressure [46].

From injury onset, the human body starts wound healing, a complex and highly regulated process [47] that requires dynamic interactions between several components, such as cells, ECM and soluble factors (like growth factors and cytokines), in order to repair and reconstruct the lost cutaneous tissue. Although this process is dynamic and continuous, it may be divided into 4 well-orchestrated events [43], schematically represented in **Figure 1.2**: i) coagulation and hemostasis; ii) inflammation; iii) proliferation; iv) wound remodeling with scar tissue formation. Although these events are sequential, some of them may overlap [48]. Wound healing involves a cascade of accurately regulated phases that correlate with the appearance of several types of cells in the wound site. The transition between phases is tightly regulated by wound healing mediators produced by several cell types. While acute wounds proceed orderly and timely throughout these phases, the process in chronic wounds may be interrupted, usually in the inflammatory phase, leading to re-epithelialization failure.

- **Coagulation and hemostasis phase** [49]: This phase, mediated by platelets, takes place immediately after injury, and its principal goal is to stop bleeding in order to prevent the blood loss by forming a fibrin clot, which also loosely joins the wound edges, limiting the spread of microorganisms within the body. A second objective, in the long-term, is to provide a provisional matrix for migrating cells that will arrive in later stages. There is vasoconstriction and the coagulation cascade is activated, leading to platelet aggregation, forming a clot. Platelets release soluble factors that activate and attract neutrophils [50], acting as promoters in the wound healing cascade. Later, these soluble factors also attract macrophages [51], fibroblasts and endothelial cells to the wound site [52, 53]. Platelets also contain molecules that cause vasodilation and increase vessels permeability, leading to fluid extravasation into the tissue, resulting in an edema;

- **Inflammatory phase** [54]: This phase aims to establish an immune barrier against invading microorganisms. In the early inflammatory phase, neutrophils infiltrate into the wound site. They start the key task of phagocytosis [55] in order to destroy and remove foreign particles, bacteria and damaged tissue, thus preventing infection. After completing their task, neutrophils are eliminated. In the late inflammatory phase, macrophages appear in the wound and continue the process of phagocytosis. Both neutrophils and macrophages are attracted to the wound site by a set of chemoattractive agents;

- **Proliferative phase** [56]: In this phase, fibroblasts, attracted by factors released by platelets and inflammatory cells, migrate from the surrounding tissues to the wound site and deposit newly synthesized ECM to replace the provisional matrix composed of fibrin [57] and fibronectin [58]. Then, fibroblasts change to their myofibroblast [59] phenotype, contracting the wound, which reduces the total area to heal. Fibroblasts synthesize collagen, providing integrity and strength to the tissue. Angiogenesis is also stimulated in this step and new blood vessels are formed, forming a vascular supply to the wound [60]. There is granulation tissue formation (connective tissue, highly vascularized and cellular). Epithelial cells also start migrating across the defects and adhere, initiating the formation of new epithelium (re-epithelialization);

- **Remodeling phase** [61]: As the wound healing final stage, this phase is responsible for the new epithelium and final scar tissue formation [43]. Although scar formation is a natural process in wound healing, the regeneration of the damaged skin without scar formation is the ultimate goal of the wound healing process. This phase is precisely regulated by some regulatory mechanisms in order to maintain a balance between collagen degradation and synthesis. The maturation phase is long, being that the new tissue is in continuous remodeling in order to mimic the maximum as possible those properties of the native tissue. Although the initial collagen deposition is quite disorganized, the new collagen matrix becomes more oriented and crosslinked over time, but the original tissue strength and native properties can never be regained. Owing to interactions between fibroblasts and the ECM,

the connective tissue shrinks, bringing the wound margins closer together. With time, capillaries stop growing, leading to a decrease in the blood flow and in the metabolic activity at the wound site. As the wound heals, macrophages and fibroblasts suffer apoptosis. The final result is a fully matured scar with a high tensile strength and a reduced number of cells and blood vessels [43]. A fibrous tissue replaces normal skin after an injury.

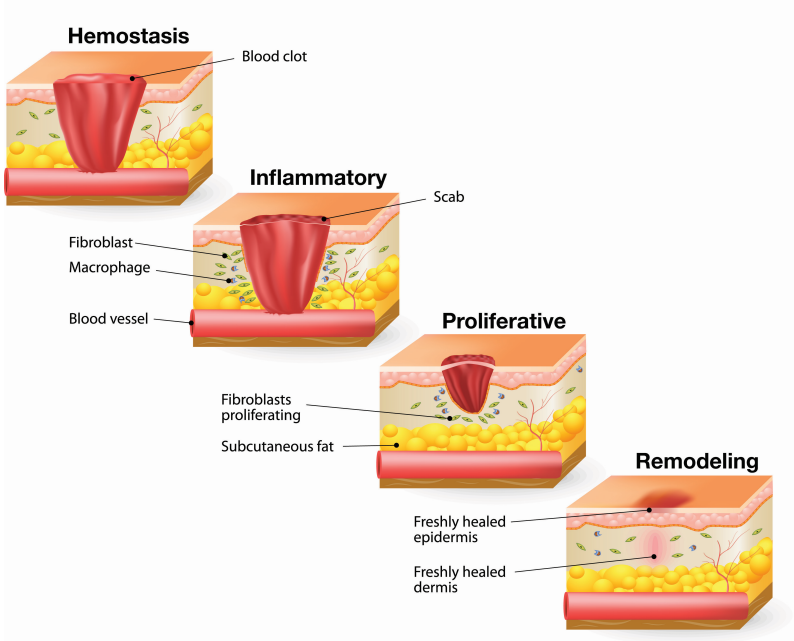

Figure 1.2: Schematic representation of the 4 stages of the wound healing process. Image from the website[1].

1.5 Context and Motivation: The Problem of Wounds, Need of Products, and Market

As already demonstrated, skin is an extremely important organ. But due to its continuous exposure to the external environment and its large surface area, it is extremely vulnerable to suffer many types of lesions, including ulcers, burns, and wounds. Chronic wounds and extensive skin loss remain a remarkable challenge in skin treatment, because the ability to self-renew of the skin is limited in deep injuries such as those [62, 17, 63].

The regeneration of functional skin remains a great challenge [64], because of the complex and multilayered structure of skin, with the presence of diverse cell types in an organized and dynamic way within the ECM. As it was previously described, the treatment and healing of skin lesions is a complex process, involving a well-orchestrated cascade of overlapping events. Although human skin has an intrinsic capacity to the self-regenerate after damage, this ability may be compromised

[1] http://www.shieldhealthcare.com/community/popular/2015/12/18/how-wounds-heal-the-4-main-phases-of-wound-healing/

due to several conditions, such as chronic wounds, deep burns, nonhealing ulcers, extensive skin loss, and diabetes [65]. An inadequate healing may lead the wound to enter in a chronic state, and thus considerably affecting the patient's health. To protect the wounds, stimulate the healing process, approximate the new skin properties to the native skin, and reduce the scar formation, several therapies and wound care products have been developed and are under constant investigation to develop new products.

The treatment of skin lesions is a pivotal issue in healthcare, requiring the need to consider several parameters that affect the healing process, such as for instance the wound type, the wound depth, the patient's health, and the level of exudate [65, 66].

According to data from the United Kingdom, it has been estimated that around 3500 people out of a population of 1 million people will be living with a wound [67], from which approximately 15% of wounds remain unresolved 1 year after presentation, resulting in a prolonged, yet avoidable, burden to patients and health systems [67]. In 2013, skin diseases resulted in direct health care costs of $75 billion (around 64 billion €), while in 2006 this number was significantly smaller (approximately $29 billion, around 24 billion €) [68], and it is estimated that this value continues to grow (which was verified). The prevalence of most skin diseases increases with overweight [69] and age [70]. As a consequence, and as a result of demographic trends, such as the increasing of life expectancy and sedentary lifestyle, the number of people with chronic wounds is growing and it is likely to continue to grow [67].

Many resources are spent on wound management. Some studies suggest that between 25 and 50% of hospital beds are filled by patients that require some form of wound treatment and management, and it is estimated that this procedure rises for over 50% of community nurse time in Europe [67]. Costs and prevalence of skin diseases are comparable with other diseases with significant public health concerns, such as cardiovascular [68]. A continued rise in the economic, clinical, and social impact of wounds [69] opens perspectives to new investments in wound care and related research and products. According to the *Markets and Markets* Website [2], the value for the wound care market in 2019 was $19,8 billion (around 17 billion €) and the forecast for 2024 is $24,8 billion (around 21 billion €).

Losing the skin barrier can bring severe consequences, such as fluids or proteins loss, cell death, or infections. Depending on the damage type, extension, and depth, the skin's innate and spontaneous healing ability may be dramatically reduced, because this ability is not unlimited. In larger wounds, the self-healing ability of the skin is insufficient to restore its native architecture, structure, composition, and function. Therefore, it is important to find and develop skin products that are able to protect the wounds and to improve the wound healing process of the native skin tissue. Furthermore, skin 3D models can be used in pharmaceutics or cosmetics industries, to investigate and test new commercial products, in which more efficient and cheaper alternatives are needed, and also minimizing animal experiments, acting as *in vitro* testing models. Skin substitutes can also be used for diagnostic purposes. More specifically for this work, wound dressings are extensively needed, as the numbers described previously clearly demonstrate.

[2] https://www.marketsandmarkets.com/Market-Reports/wound-care-market-371.html

1.6 Main aims of the thesis

The main goals of this dissertation includes:

1. Design a double network wound dressing based on natural polymers that can be used for the management of skin wounds by providing protection to the wound bed and stimulating the healing process;

2. Characterize the key properties of the wound dressing and evaluate their potential for subsequent biological evaluation.

The wound type to address depends on the properties that will be achieved in the production of the dressings.

1.7 Outline

Chapter 1 consists in an introduction, containing a background on skin, the context and motivation of the work, and the goals to achieve in the thesis.
Chapter 2 focuses on the state-of-the-art of current strategies for wound management. Wound dressings will be reviewed in more detail as they are the focus of this research project.
Chapter 3 describes the specific goals of the research project.
Chapter 4 presents the materials and methodologies used during the experimental work.
Chapter 5 details the obtained results and discusses the findings of the project.
Chapter 6 includes the conclusion, final remarks and future work.

Chapter 2

State of the Art

In this Chapter, most commonly used therapeutic options for skin lesions treatment and reconstruction will be addressed. Current approaches, their techniques, technologies, mechanisms, advantages, disadvantages, and applications, as well some types of biomaterials and cells, will be addressed in **Section 2.1**. Examples of products available in the market will also be provided. Then, the focus will be on the materials and techniques used in this project to produce wound dressings (**Section 2.2**).

2.1 Techniques and biomaterials used on wound treatment

2.1.1 Traditional therapies

Nowadays, and despite the recent advances in wound care products, there are several conventional therapies based on compounds from natural origin, such as plants, animals or minerals [64], that constitute good alternatives to promote wound healing. Natural compounds have been used in wound treatment for many years, owing to their therapeutic benefits, such as antimicrobial and anti-inflammatory characteristics, as well as cell-stimulatory properties [64]. These therapies bring some advantages for skin wound treatment when compared to modern products and therapies, allowing to overcome some of their drawbacks, including the long manufacturing time, expensive costs, and the increase of bacterial resistance [64], enlarging the access to healthcare. In fact, there has been a progress on the development of new extraction and purification methods, treatment, and quality control assessment protocols, but some parameters as safety, side effects, and efficiency of these compounds need further research [64], because the use of some natural therapies is supported by popular wisdom rather than by scientific evidence. Beyond the uncertainty of some features of the natural compounds, they can exhibit batch-to-batch variation and generally depend on the location and season [64], making the standardization of the involved procedures (e.g. extraction, manufacturing, and treatment protocols) difficult and this is an absolutely essential requirement. There are many ongoing investigations on the clinical safety, efficiency and side effects of traditional therapies. Despite the advances, further investigation is needed toward approval of natural healing compounds and traditional therapies, to allow their entrance into the

national healthcare systems [64]. Usually, the use of this type of therapies is considered safe, not presenting significant adverse reactions. Nevertheless, not few times the contact between the compound and wound may result in pain for the patient, contact dermatitis, skin irritation, itching, or erythema associated to topic solutions, or vomiting and diarrhea associated to oral administration.

- **Herbal-derived compounds**: The most popular and commonly used traditional products for the skin wounds treatment are herbal-derived compounds. These therapies include the application of herbs, herbal preparations, and herbal products, containing biologically active compounds that stimulate the healing process. Nowadays, a substantial variety of plants are being investigated and used for skin wound treatment [71]. Products based on herbs can be applied as creams, extracts, ointments, and emulsions, and can be administrated through oral, topical, or systemic routes. Some of these products have also been incorporated within dressings or nanoparticles in order to achieve a more controlled release and improve their stability [72]. *Aloe Vera* [73], a cactus-like plant, is the most popular herb among the ones used in wound healing.

- **Animal-derived compounds**: Products of animal-origin, as the herbal ones, have been used for centuries, due to their therapeutic properties. These treatments are largely available, being honey and *propolis* two representative examples [64].

- **Living organisms**: Living organisms have been widely used in the last years for wound healing. Maggots [74, 75, 76], the larvae of the fly, are one of the most used living organisms, presenting an exceptional ability to stimulate wound debridement and a good antimicrobial activity, being directly applied to the wound site.

2.1.2 Solutions, creams, and ointments

Liquid and semi-solid preparations have also been widely used for wound bed decontamination and preparation, disinfection, pain-relief, and wound healing, acting as agents with antimicrobial and cleaning [77, 66]. However, due to their physical form, these products have a limited capacity of permanence in the wound site for the necessary period of time [78]. In order to overcome this problem and to facilitate their handling, most of these products have been embedded or incorporated within wound dressing [66]. These products, also due to their topical nature, have been more widely used for epidermal regeneration.

2.1.3 Wound dressings

When a skin lesion involves the loss of large amounts of tissue, the immediate covering of the wound is required, in order to protect it and preventing infection and dehydration. Wound dressings (**Figure 2.1**) are the used products for this end, and have been widely used owing to their relatively low cost, ease of use and effectiveness to clean and protect the wound from the external environment [66]. Wound dressings are widely used for wound treatment, providing protection

of the wound from infection in an initial stage of the healing, while in some wounds can also be used to perform additional functions, such as acting as drug delivery systems, incorporating therapeutic bioactive agents and releasing them into the wound bed, or even hydrate the wound bed [66, 79, 80, 73]. Some wound dressings may also provide an adequate 3D biological support that enables cell colonization, migration, proliferation, and even stimulates new tissue formation [81]. Depending on the type and extension of the wound, different wound dressing may be applied. There is a huge number of wound dressings commercially available, in the form of thin films, gels, or foams [78, 66, 82], having different applications.

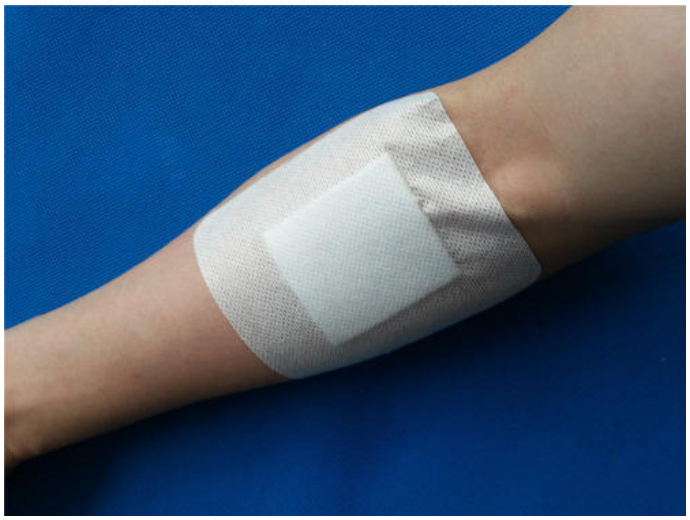

Figure 2.1: Wound Dressing. Application of a wound dressing on the skin. Image from the website[1].

Wound dressings are usually classified into traditional and modern dressings [78]. Bandages, cotton wool and gauzes are examples of often used traditional dressings [78, 66] for treatment of skin lesions, providing a protective barrier against microorganisms. However, traditional dressings have some problems. Once applied to the wound, these products may absorb high volumes of fluid, leading to wound bed drying, which may result in inhibition of the healing process, because moist has a main role on the healing process [83]. Additionally, these dressings may strongly adhere to the wound bed, due to the high absorption of exudate, making its removal difficult. Owing to these limitations of traditional dressings, they are typically combined with other products or dressings or applied as secondary dressings, in order to absorb exudates and protect the wound from the entrance of pathogens. Modern dressings were developed to address the limitations of traditional ones [66], being able to maintain a moist and warm environment, providing favorable conditions for the healing process [78, 84], such as proliferation and migration, while absorbing the excess of exudate [85]. They are designed to cover the wound in order to prevent infection and skin desiccation. Unlike the traditional dressings, modern ones are more flexible, in order to not adhere so strongly to the lesion site.

[1] https://www.indiamart.com/proddetail/sterile-wound-dressing-19081917348.html

There are several natural and synthetic biocompatible polymers with adequate biocompatibility that can be applied for the development of wound dressing materials [82]. A combination of both types is also used: combining materials to achieve superior mechanical and biological properties is an effective strategy to create dressings that perform better than single-material dressings. One example is the formation of chitosan-pectin polyelectrolyte complexes (PECs) and alginate-pectin PECs for wound dressings, which present better biological properties than wound dressings composed of a single polymer, such as accelerated healing of incision wounds in a rat model [86].

An ideal wound dressing should fulfill key requirements [66, 79, 87, 85, 73, 88, 89]:

- Biocompatibility;
- Protect the wound from infection, by providing a protective barrier against microorganisms and being resistant to bacterial invasion;
- Be non-antigenic, non-toxic and non-allergenic;
- Present adequate physical properties, including flexibility to fit the lesion region, and exhibit good adhesion to the wound bed (but not strong adherence that may difficult its removal). The dressing must be both robust and flexible to allow adherence to the wound surface, while maximizing patient comfort, being easy to apply and remove without trauma;
- Present adequate mechanical properties, such as great porosity with adequate porous size, an adequate surface morphology, and maintain its integrity during use;
- Provide thermal insulation;
- Hold an excellent swelling ability, quickly absorbing or remove the excess of wound exudate, but without completely drying the wound site, which would inhibit the healing process;
- Have an optimal water vapor transmission rate (WVTR) to maintain a moist environment of wound site, controlling water loss at the wound site, ideal to promote wound healing;
- Allow gaseous exchanges, promoting effective oxygen exchange and being permeable to water vapor;
- Provide a moist and warm environment to the wound bed, which are favorable conditions for the healing process;
- Promote wound healing, accelerating tissue regeneration to promote fast wound closure, and minimizing scar formation.

These properties have to be assured in order to produce a wound dressing with high quality and applicability.

2.1.4 Skin grafting: Autografts and allografts

When the lesion extension reaches deep into the dermis and/or affects large areas, a more complex treatment is needed [66].

- **Autografts**: Autografts are currently the golden standard in skin regeneration for partial- or full-thickness skin wounds [66, 90], and consist on the transplantation of a piece of tissue from one body part to another one, in the same individual. Skin grafts are harvested from undamaged skin areas and placed on the full-thickness wound. Afterwards, the newcomer tissue and the wound capillaries start merging together. Autografts provide pain relief, own good adhesion to the wound bed, restore all structure and functions, and carry little risk of rejection. However, as there is a limited availability of donor sites in the human body, it may trigger an induction of a hypertrophic scar formation both on donor and in the recipient site, the hospital stays are long, and the pigmentation may be different comparing the newcomer tissue and the engaging tissue [66, 90];

- **Allografts**: Allografts, usually obtained from skin banks [90], are another possible procedure, but are usually used as a temporary prevention of contamination and fluid loss of the wound [66, 90]. These grafts may be incorporated into deep wounds and are off-the-shelf (i.e., they are immediately available to cover the wound and do not need to be specifically made to suit a particular purpose). However, there is also limited donor availability from banks. Allografts bring some safety issues associated with immune rejection and disease transmission, as well as some ethical issues [91, 92, 93].

2.1.5 Bioengineered skin substitutes

Tissue engineering (TE) is a multidisciplinary field of research that has been extensively explored to develop biological substitutes to restore, maintain, or improve the function of skin [3, 66, 94]. It involves the combination of cells, scaffolds, and/or biomolecules such as growth factors [66]. Skin regeneration is a field where TE commercial products are already available and under clinical utilization [66].

Skin grafts and other skin treatments explored previously, still have significant limitations for skin regeneration, despite the considerable advances in manufacturing processes and in the biomaterials used. Traditional products are not able to replace lost tissue, autografts have the problem of the limited amount of donor skin, and allografts may present severe risks, such as disease transmission or immune rejection [17, 62, 95, 63, 96]. In order to address these limitations, bioengineered (or tissue-engineered) skin substitutes were developed. These substitutes are 3D wound care products to treat burns, ulcers, deep wounds, and other lesions, based on cells, biomaterials, or their combination [84]. Bioengineered substitutes use principles of TE in the development of cellular and acellular substitutes for epidermis, dermis, or both. They have several functions, including protecting the wound from fluid loss and contamination and reduce inflammatory responses and

scarring, which leads to a decrease in hospitalization time and in the number of surgical procedures [17, 62, 97, 63]. These substitutes usually consist of a temporary or permanent scaffold material that works as a template for cell adhesion, differentiation, and proliferation, guiding new tissue formation [97, 96]. Bioengineered skin substitutes help in skin repair and regeneration, by improving healing rates, and restore the efficient properties of skin when lesions occur [98, 63].

Bioengineered skin represents artificial replacements alternative to skin grafts [17, 99], being the most successful TE products so far. The ideal skin substitute must have the capacity to resist infection, resemble the physiology of normal skin, have long-term stability, be easy to prepare, store and use, withstand wound hypoxia, be effective in achieving tissue regeneration, be cost efficient and widely available, and promote re-epithelialization and revascularization [62, 17, 63, 96, 100]. However, no ideal skin substitute has been developed yet [63].

The major goal of TE constructs is to act as skin equivalents, restoring the functional properties of skin and facilitating repair and regeneration [63]. A skin substitute has 3 important components: cell, tissue-regeneration inducing factors (such as growth factors and cytokines), and matrix or scaffold [17].

Skin substitutes can be classified according to different criteria [63, 96]:

- **Anatomical structure** [66, 63]: epidermal substitutes (substitutes consisting on cultured epidermal components only), dermal substitutes (those with only dermal components), and dermo-epidermal substitutes (substitutes containing both epidermal and dermal components, usually used to replace lost dermis in the treatment of full-thickness lesions, affecting both epidermis and dermis).

- **Type of biomaterial** [95, 98, 63, 99, 101]: biological (tissue-engineered skin from natural skin, using for example cells or decellularized ECM to build a new skin substitute), synthetic (artificially made in order to mimic the natural characteristics, not presenting the risks of immune rejection and disease transmission), or biosynthetic (composite of natural and synthetic materials). Biological substitutes may be autografts (uses skin from the own patient, being that the samples or grafts can be harvested from different parts of the body), allografts (uses components from another individual, alive or cadaver, such as cells or decellularized ECM, but in emerging situations it is difficult to obtain a fresh allograft, and there is a greater risk of immune rejection or disease transmission), or xenografts (uses skin from other species, i.e., non-human tissue, mainly porcine and bovine origin, being also susceptible to be rejected by the host immune system or transmit a disease). Typically, both allografts and xenografts must be later replaced by autografts or other substitutes, to avoid immune rejection [102, 96].

- **Cellular component** [95, 17, 98, 63]: cellular constructs (contain living cells that may incorporated in biomaterials that function as scaffolds) and acellular or cell-free constructs (without cells, it is made of a matrix or scaffold, and if the biomaterial source is natural, the cells have to be removed, i.e. decellularized scaffold).

- **Duration of the cover** [98, 63]: permanent or temporary.

The most widely used strategy is to use biomaterials with co-cultures of skin cells in order to support and promote cell adhesion, cell proliferation, and ECM deposition [62]. Human fibroblasts and keratinocytes (dermal and epidermal components, respectively) are collected from the patient through a biopsy, isolated from each other, and then cultured in an appropriate medium and expanded *in vitro* [62, 17]. This procedure may take approximately 3 weeks. As a result, the wounds have to be treated with temporary dressings that protect the lesion and stimulate the healing [103, 66]. Keratinocytes, the main epidermis cells, form a stratified epithelium. Fibroblasts are the main cells of the dermis and produce remodeling enzymes that have a major role on the wound healing process. Fibroblasts also play a role in acute wound contraction, in skin remodeling, and in stimulating keratinocyte growth by secreting soluble factors [62, 104].

Some commercially available bioengineered skin substitutes exist and are usually grouped by the type of substitute (epidermal, dermal and dermo-epidermal), the graft type (cell-based or cell-free), the cell source (autologous or allogenic) and the matrix or scaffold used (if it is used; they may also be scaffold-free) [66, 95, 63, 98, 105, 106, 93, 96]. These products have different functions and actuation mechanisms, and are used in different types of wounds (according to the cause, width and depth). Commercially available skin substitutes are mainly composed of natural materials (e.g., collagen, elastin, fibrin, and hyaluronic acid), with only a few of them using synthetic materials (e.g., silicon, PGA, and PLA) [17, 96]. Due to difficulty to produce and sell autologous skin equivalents, most of the available commercial tissue-engineered substitutes consist of biomaterial-based matrices containing allogenic cells, usually derived from neonatal foreskin, which are known to present robust metabolic activity, reduced risk to being rejected and are a tissue source of stem cells [17].

In spite of being the most advanced and sophisticated available products for skin wound treatment, bioengineered skin substitutes have some limitations, such as high manufacturing costs and time, poor integration due to deficient vascularization, scarring at graft margins, inefficient adhesion to the wound bed, mechanical weakness, the lack of skin appendages (because they usually comprise only two types of cells, keratinocytes and fibroblasts), and variable engraftment rates [103, 66, 17, 63, 1, 96]. Furthermore, most of the available products are found in limited quantities and at high prices [62]. To overcome the long fabrication times associated with the epidermal substitutes, the cells obtained from the biopsy can be directly sprayed into the lesion, allowing the local delivery of epidermal cells [103, 66], thereby resulting in a faster epithelialization and epidermal maturation. The ambitious goal is to fabricate a dermo-epidermal substitute that rapidly vascularizes and supports a stratifying epidermal graft [62, 96], is easy to handle, relatively inexpensive, able to mimic the normal skin physiology and is cytocompatible to avoid immune rejection. The standardization of processes in bioengineered skin substitutes production would be an important advance, improving the control over the processes and increasing large-scale production [62]. Other important advances would be the increase of the substitutes lifespan, the inclusion of additional cell types to add skin appendages and the possibility to stimulate angiogenesis in 3D

models. This stimulation of angiogenesis in 3D may be achieved by the use of bioreactors that provide the simulating environment needed to develop blood vessels in a mature state [62, 107, 96]. Although there exist some skin substitutes with great complexity, further research is needed towards the improvement of their micro-architecture and large-scale availability [62]. However, in spite of the still needed refinements, bioengineered skin substitutes are a very promising approach to treat skin wounds. A key point is to know how faithful is the skin substitute in relation to the normal skin state [95]. In summary, controlled clinical trials are required, involving a significant number of patients, to confirm their clinical effectiveness.

2.1.6 Biophysical therapies

Biophysical technologies are usually used to improve the wound healing outcomes, and include the following therapies [84, 108]:

- **Ultrasound therapy**: Uses ultrasound waves to facilitate wound healing;
- **Electrical stimulation**: Uses exogenous electrical current to facilitate wound healing;
- **Phototherapy**: Uses energy from the electromagnetic light spectrum to stimulate wound healing;
- **Negative pressure wound therapy**: Creates a closed system into the wound, preventing fluid loss and promoting faster wound healing.

2.1.7 TERM strategies and biofabrication technologies

2.1.7.1 Bottom-up and top-down approaches

Although being clinically effective, currently available skin grafts, wound dressings, and bioengineered skin substitutes are still characterized by presenting some important constraints, including high costs, limited adherence to the wound bed, patient morbidity, inadequate vascularization, and the inability to reproduce skin appendages [66, 109]. Advanced TERM strategies provide a permanent, effective, and viable alternative to address and resolve these limitations, by the combination of cells, biomaterials, growth factors, and biomanufacturing techniques [66, 93] for the fabrication of constructs mimicking skin anatomy and physiology and promote its regeneration. Two different approaches can be considered: bottom-up and top-down approaches [66, 110, 2, 109], represented schematically in **Figure 2.2**, and both of them can be controlled by computer, thus permitting some processes to be automated, such as cell deposition and seeding, and scaffold fabrication, allowing the production of well-organized 3D multilayer skin substitutes containing skin cells. The main difference between the two approaches consists in the use or not of a scaffold as supporting material [66, 2, 109] to produce engineered tissues.

- **Top-down approaches**: Also known as scaffold-based approaches, are the most classical approach, involving seeding cells into biocompatible, biodegradable and porous scaffolds,

which provide a substrate and physical support for implanted cells, guiding new tissue formation, to form tissue-engineered constructs [66, 73, 111]. Scaffolds may be natural or synthetic, and may be produced by conventional strategies or through additive manufacturing techniques, while cells may be autologous or allogenic. The scaffold containing cells is cultured *in vitro* to maturate, and then it will be implanted in the lesion site. The cells adhere to the scaffold surface, proliferate, and secrete their own matrix, stimulating the formation of new tissue, and then the scaffold may degrade at a certain rate after implantation. Growth factors may also be added. The maturation of the assembly may be done in a bioreactor [110, 66, 1]. Although this approach allows good interactions between cells and scaffold, and satisfactory control over the scaffold features (especially when biomanufacturing techniques are used), it presents some drawbacks, such as heterogeneous cell distribution, low cellular density, diffusion limitations and slow vascularization, which can lead to heterogeneous tissue growth and deficiency in resembling the complex cellular organization of the native skin at the micro- or nanoscale [1]. Ideal scaffolds for skin regeneration must guide tissue formation, prevent scarring, and retain cells and growth factors.

- **Bottom-up approaches**: Also known as scaffold-free, this approach provides the capacity to address limitations of the top-down approach. It produces engineered tissues without using scaffolds as supporting matrices, as it involves automated assembling of small preformed fabrication units containing cells (such as cell aggregates, cell sheets and spheroids) placed in specific 3D locations to form larger, complex and organized 3D constructs resembling the structure and functions of human tissues. These constructs can be produced by different biofabrication techniques, such as bioprinting, microfabrication, bioassembly (used to create hierarchical constructs with a prescribed 2D or 3D organization), self-assembled aggregation, cell-laden hydrogels, or microfluidics [66, 109, 1, 111]. Usually, these approaches comprise 3 key elements: a bioink containing the cell suspension to be printed, a temporary support for the deposited cells, and a bioprinter or dispenser. Bottom-up approaches have potential to create a vascular structure in 3D constructs through the fabrication of a vascular tree inside the structure. Additionally, it also allows the deposition of multiple cell types with 3D organization, which may be very important for skin regeneration, as previously described [1]. Cells have the ability to aggregate. The main limitations of scaffold-free strategies are that some cells cannot establish cell-cell junctions, migrate or produce sufficient ECM [109].

2.1.7.2 Bioprinting technologies

Recent advances in additive manufacturing have enabled the tri-dimensional printing of cells, biomaterials and supporting components, into functional and complex 3D living tissues. Biofabrication is a multidisciplinary field that combines the principles of biology, materials science and engineering, for the automated fabrication of biologically functional constructs, using living cells,

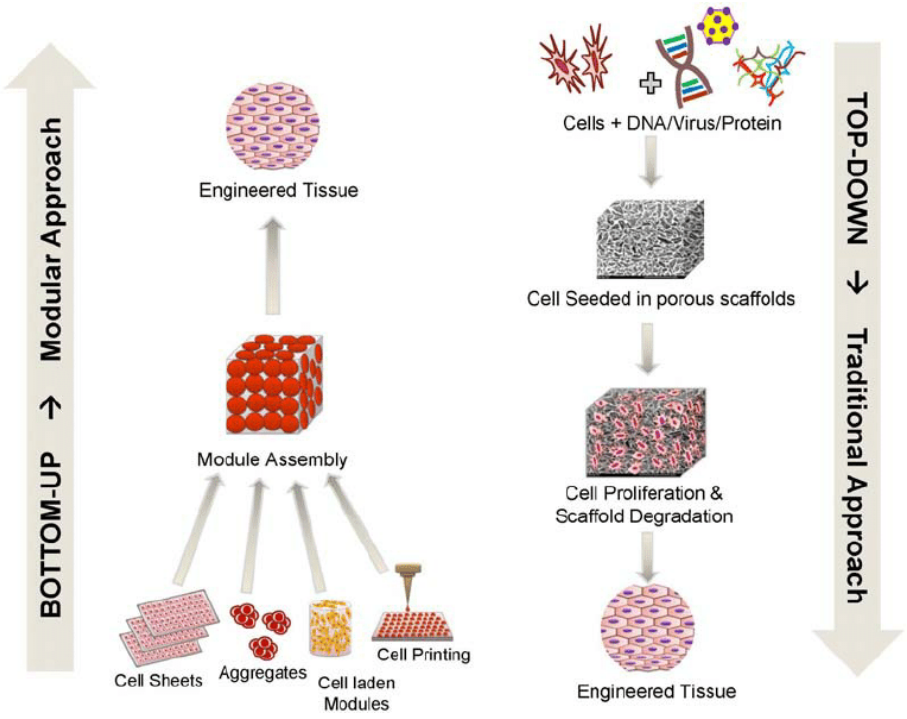

Figure 2.2: Bottom-up vs. top-down approaches. Representation of TE bottom-up and top-down approaches. Image from Annamalai *et al.* (2014) [2].

biomaterials, and bioactive molecules, through 3D manufacturing techniques, and it is being applied in the production of biological structures for TE and RM applications [66, 112, 1, 3]. Biofabrication is the use of additive manufacturing approaches for TERM applications, as schematically represented in **Figure 2.3**.

Bioprinting, one main strategy of biofabrication, relies on the printing of single cells or cell aggregates, bioactive molecules, and biomaterials, into complex and well-organized 3D scaffolds, layer-by-layer, from a computer-aided design model, in an automatic way, with high resolution, accuracy, reproducibility, with spatial, orientation, and concentration control, to build constructs analogous to human tissues with intricate architectures, to promote the synthesis of skin both *in vitro* and *in vivo* [66, 112, 113, 110, 1, 114]. Bioprinting provides precise control over the porosity, pore shape and size, and pore interconnectivity [66, 110], while offering the possibility of printing heterogeneous constructs. Bioprinting usually involves one of two approaches: printing constructs and then seed cells or print constructs containing cells (more interesting but more complex process in terms of parameters, and often requires a maturation step).

Bioprinting involves additional issues comparing to the printing of non-biological constructs, including the choice of biomaterials, cell types, and biomolecules, and additional technical matters regarding the construction of tissues and the sensitivity of live cells. There are many additive manufacturing techniques, but not all of them can be used to print tissues because they do not offer adequate properties and conditions for cell viability, mainly due to high temperatures that kill the cells, or the use of crosslinking agents or organic solvents that are incompatible with live

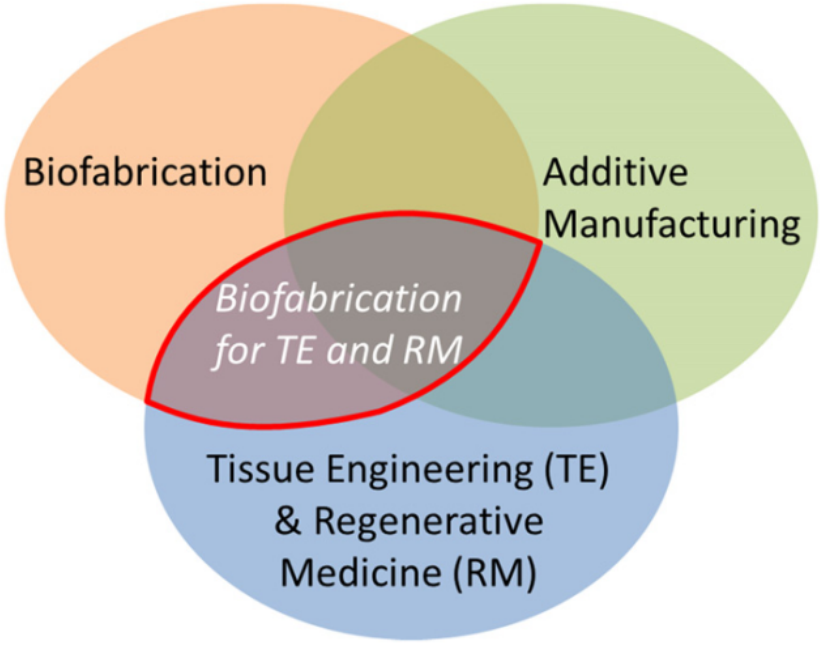

Figure 2.3: Schematic representation of the relation between biofabrication, additive manufacturing and TERM. Image from Groll *et al.* (2014) [3].

cells. Addressing all these complex issues requires the integration of knowledge and techniques from biology, materials science and engineering [112], in order to find or develop biomaterials that not only provide the desired mechanical and functional properties for the construct, but also are compatible with live cells and with the printing process.

Understanding the composition and organization of the components of a functional tissue is an essential step to reproduce its complex and heterogeneous architecture. To acquire information on this topic, medical imaging is an indispensable tool. Computed tomography (CT) and magnetic resonance imaging (MRI) are the most common examples. Computer-aided design (CAD) and computer-aided manufacturing (CAM) tools are used to generate computer-based 3D models of a tissue architecture and to control the printing process [112, 110, 1]. The bioprinting feature of precisely pattern structures combined with medical imaging and computer-controlled printing techniques allow generating specific constructs for each patient or application, replicating the intricate composition and architecture of the skin [1, 3]. After having the image, a design process is carried out, followed by materials and cell selection. Finally, the construct is printed and then used on the desired application.

Bioprinting technologies, which allow direct incorporation of cells in the printing process, can be broadly classified according to the printing principle, into 3 main categories: light-based technologies, extrusion bioprinting, and inkjet bioprinting [66, 1]. On these techniques, the material to be printed, known as bioink, is loaded in a reservoir or coated in a ribbon and subsequently deposited onto a receiving substrate through the action of some mechanisms, such as vibration, pressurized air, light, and thermal or mechanic effects [1].

- **Vat photopolymerization**: Produces solid constructs in a layer-by-layer fashion through the selective photoinitiated curing reaction of a liquid photosensitive material containing a low-molecular weight prepolymer, photoinitiators and additives, present in a vat [66, 110]. This curing reaction is induced by the incidence of light with appropriate energy, duration, intensity, and wavelength. The supplied energy must be sufficient to bond a substantial number of molecules, forming a highly crosslinked polymeric structure [66].

- **Laser-assisted bioprinting**: Laser-assisted biorpinting (LAB) is a light-based technology that involves the incidence of a highly-energetic pulsed laser beam to generate bioink droplets, leading to the local ejection of small droplets that fall towards a receiving platform [112, 1].

- **Inkjet bioprinting**: Bioprinting technology based on the dispensing of controlled volumes of a liquid bioink, generally small drops, onto a collector substrate [112, 1]. According to the mechanism responsible for droplet formation and ejection, inkjet bioprinting can be divided into thermal and piezoelectric inkjet. In thermal inkjet bioprinting, the print head is electrically heated in order to produce short pulses of pressure that generate a bubble that expands and ejects a controlled volume of fluid bioink as a single droplet through the nozzle [112, 1]. Several studies have showed that this localized heating, due to the reduced pulses duration, do not imply considerable consequences on the viability, post-printing function, and stability of cells and biomolecules [112, 1]. In piezoelectric inkjet bioprinting, the mechanical deformation of a piezoelectric transducer controlled by the application of an external voltage allows the generation and ejection of uniform droplets. The liquid is broken into droplets at regular intervals due to the waves created by the piezoelectric crystal [112, 1]. Here, cells are not exposed to heat or mechanical stress, and there is a formation of droplets of uniform size.

- **Extrusion bioprinting**: Most widely used strategy for the fabrication of heterogeneous cell-laden 3D constructs in TE. Extrusion-based processes comprise reservoirs containing the bioinks (materials and cells), a moveable nozzle controlled by a CAD system, and a platform that collects the printed material, in order to form the solid construct, in a layer-by-layer and automated process [66, 113, 1]. The bioink may be cell-laden polymeric solutions, cell suspensions, decellularized ECM components, microcarriers, or tissue spheroids. Typically, cells suspended in either culture medium or a polymer solution are loaded into syringes and dispensed through a nozzle, being printed onto a receiving platform by mechanical forces generated by a rotating screw or a piston, or by pressurized air [113, 110, 1]. The bioink leaves the extruder in the liquid state and stiffens instantly. The previously formed layer is the substrate for the next one, so it must be maintained at a temperature just below its solidification temperature, in order to assure good adhesion between consecutive layers [110]. Being able to control and regulate the temperature of both the platform and the bioink during the printing process is very important to manage the viscosity of the bioink and to induce *in situ* gelation of polymers sensitive to temperature.

- **Electrospinning**: Simple and versatile electrostatic fiber fabrication technique used to produce random fiber meshes mimicking the micro- and nanostructure of the natural ECM for TE applications [115, 111, 116]. It results in the production of nanofibers with diameters ranging from a few nanometers to several micrometers [66, 116]. Electrospinning is a strategy commonly used for the fabrication of 3D constructs at the nano-organization of ECM components, that function as scaffolds for skin regeneration, providing an adequate microenvironment for cell adhesion, proliferation, and differentiation [66, 110]. In the biomedical field, among other applications, electrospinning has been used for the production of wound dressings and scaffolds for TE applications [115, 117, 118, 111]. Nanopatterned electrospun fiber meshes hold great attractiveness for TE applications and skin regeneration due to their similarity to the meshwork of dermis ECM fibers, high surface-to-volume ratio, porosity, and interconnectivity, which allows cell migration, tissue ingrowth, as well as the incorporation of molecules for controlled delivery [115, 66]. A common electrospinning apparatus consists on a high voltage power supply, electrical wires, a capillary tube with a needle and a collector. The polymeric solution is contained in the syringe and is pumped at a controlled rate. The voltage is turned on, the surface tension of the polymer is overcome by the opposing electrostatic forces and the polymer jet is initiated. During the jet's travel, the solvent is evaporated and charged polymer fibers are placed into the collector [115]. The collector can be moved in the vertical direction and in the x-y plane, enabling electrospinning to be an addictive technology [66]. In order to allow the direct incorporation of cells during the printing process, allowing better cell integration into electrospun fibers, cell-electrospinning was developed. This technique is based on the electrospinning process, but is able to fabricate fibers with encapsulated viable cells, using hydrogels. Electrospraying [119] is a similar technology to electrospinning, but instead of producing nanofibers, it produces spherical bodies (microparticles), that are deposited onto a supporting material. Most of the used devices used to produce nanofibers can be also used for electrospraying and thus for the production of nanobeads. For electrospraying, nanoparticles must be evenly distributed inside a polymer solution.

Table 2.1, presents the main bioprinting technologies, their operation mechanisms, advantages and disadvantages [66, 112, 113, 110, 1]. In **Figure 2.4** the described techniques are represented.

Polymers are the most used biomaterials in bioprinting [101]. Bioinks consist of decellularized ECM or hydrogel precursor solutions loadable with cells and biomolecules, and are very important on the quality of printed constructs and on the reproducibility of the printing process. Depending on the used bioprinting strategy, bioinks can be dispensed as continuous strands or small droplets, as already discussed, resulting in constructs with different levels of accuracy, resolution, shape fidelity and heterogeneity, and in different printing times, leading to more or less complex and

[2] https://www.lboro.ac.uk/research/amrg/about/the7categoriesofadditivemanufacturing/vatphotopolymerisation/

[3] https://www.nanoscience.com/techniques/electrospin/

Table 2.1: Examples of biofabrication technologies, their main characteristics, advantages and disadvantages

Technology	Principle of operation	Advantages	Disadvantages
Vat photo polymerization	Layer-by-layer curing of a photosensitive material	High resolution and precision	Requires post-processing with UV-light and lacking strength and durability
Laser. assisted bioprinting	Incidence of a highly-energetic pulsed laser beam onto a ribbon coated with the bioink to be printed, generating the local ejection of small droplets	Non-contact, nozzle free printing, not presenting clogging issues; high cell density, viscous bioinks; high printing speed, reproducibility and resolution	Cell-laser interaction; difficult to create constructs in the mm range; low to medium throughput; high cost
Inkjet bioprinting	Layer-by-layer drop deposition of controlled volumes of a liquid bioink onto a collector substrate	Operation with multiple printheads; non-contact printing; high printing speed and throughput; low cost	Clogging issues; limited bioink viscosity, low cell densities; possibility of cell sedimentation and agglomeration.
Extrusion bioprinting	Layer-by-layer deposition of filaments by direct contact of the print head with the collector	Ability to create constructs at clinically relevant scales; continuous printing of viscous bioinks with high cell densities; Easy integration with other biofabrication strategies; medium cost, resolution, and throughput.	Difficult to generate hierarchical 3D constructs with intricate geometries; nozzle clogging; mechanical stresses generated during the printing
Electrospinning	A polymeric solution in the reservoir forms a jet due to a high voltage supply, overcoming the opposing electrostatic forces, and it is deposited into a collector	Possibility to produce porous architectures, with high surface and interconnectivity; operational simplicity and versatility; possibility to process a wide range of materials; possibility to combine fibers with different diameters; mimic nano and microscale.	Lack of control over porosity; poor mechanical properties; nonuniform thickness; electrical field may affect cells; relatively expensive

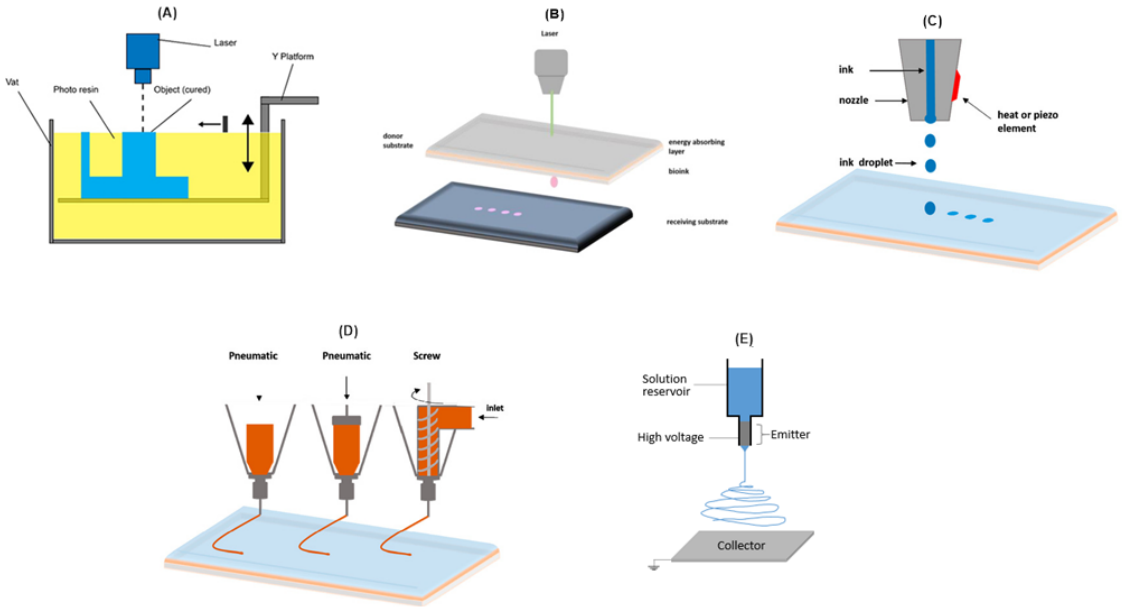

Figure 2.4: Biofabrication technologies. (A) Vat photopolymerization: laser beam focus in a photosensitive polymer (that may be photocurable resin). The solid object (cured) is created in the construction platform, that only moves in one axis. Image from the website[2]; (B) Laser-assisted bioprinting: a laser beam is focused directly on the print ribbon (donor substrate). Below the ribbon is the absorbing layer, and below it is the bioink. The bioink is deposited onto the construction platform (receiving substrate). Image from Kryou *et al.* (2019) [4]; (C) Inkjet bioprinting: a bioink present in a reservoir passes through a nozzle, suffering the action of a thermal or piezoelectric actuator. The printed droplets are dispensed into a receiving or construction platform. Image from Kryou *et al.* (2019) [4]; (D) Extrusion bioprinting: pressurized air, a piston, or a screw actuate over a bioink present in a reservoir, and the bioink passes through a printing nozzle, forming a filament that is deposited into a platform. Image from Kryou *et al.* (2019) [4]; (E) Electrospinning: the polymeric solution in the reservoir forms a jet due to a high voltage supply, overcoming the opposing electrostatic forces, and it is deposited into a collector. Image from the website[3].

intricate 3D structures. The success of the bioprinting process highly depends on the design and development of printable bioinks capable of support cell functions and stimulate the production of new ECM components, having adequate and tunable rheological and mechanical behavior, biophysical properties, biochemical composition, and biological response [1]. The development of bioinks is a complex and multidisciplinary process. The interactions between the bioink and the substrate, as well as its rheological, biophysical, biochemical, and biological properties, are important parameters determining the resolution and accuracy of printed constructs. Although recent advances have been achieved on the design of printable bioinks, the quantity of bioinks for bioprinting cellular skin substitutes is still limited.

Although promising, bioprinting strategies have limitations in replicating the heterogeneity of cells and ECM components in the natural skin [1]. Reproduce the multitude of cell types and the complex micro-architecture of the ECM components with enough resolution to recapitulate

biological functions remain the major challenge of bioprinting [112, 110]. In order to better resemble the human skin, further improvements in the bioinks design, in bioprinting technologies, and on the printing of vascularized constructs are needed [1, 5]. Standardization, reproducibility, the production of vascularized structures and the formation of skin appendages remain important challenges to overcome in bioprinting in order to reproduce the structural resolution of different tissues and organs.

In situ bioprinting is a more recent bioprinting approach that omits *in vitro* cell culture and tissue maturation steps [3]. Rather than printing 3D substitutes, seeding cells and then culture it in *in vitro* conditions for subsequent implantation, cellular skin substitutes are directly printed on the patient's lesion [1, 66]. This technique enables the immediate cover of the wound and recruiting and instructing resident cells, inducing *in situ* regenerative mechanisms. *In situ* bioprinting has great potential for clinical applications, due to the elimination of cell maturation processes and of some post-processing operations, the ability to fabricate personalized substitutes for each patient, and it may solve some issues related to vascularization *via* controlled deposition of endothelial cells, reducing issues regarding the integration on the host tissue [66].

A general representation of a bioprinting process is schematically represented in **Figure 2.5**, showing its workflow, which involves an imaging process, the design, material and cell selection, the bioprinting process, and finally the application.

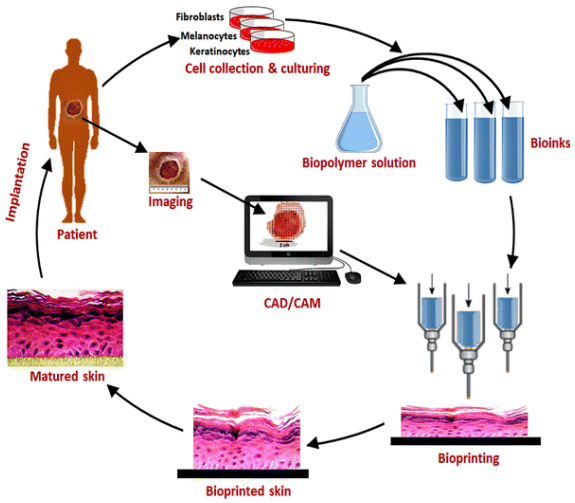

Figure 2.5: Common steps in the fabrication and bioprinting of a skin substitute. Various cell types (e.g., fibroblasts, keratinocytes) can be collected from individuals (it can be autologous, i.e. from the patient himself, or allogenic, i.e. from other person) and expanded *in vitro*. The cells are mixed within a polymer, forming the bioink that will be printed through a bioprinting approach. Characteristics of the wound are captured through imaging technologies, and a CAD/CAM system controls the printing process according to the desired form. The skin substitute may maturate *in vitro* and then is implanted in the patient. Image from Augustine *et al.* (2018) [5].

In **Tables 2.2** and **2.3** the explored approaches that were described until now, as well as their advantages and disadvantages, are presented.

Table 2.2: Skin therapies (1/2)

Technique	Advantages	Disadvantages
Herbal derived compounds	-Promote wound healing -Anti-inflammatory, antiseptic, and antimicrobial properties -Cell-stimulating properties -Variety of administration routes -Low cost and large availability -Can be incorporated within dressings or nanoparticles -Stimulate angiogenesis	-May present irritation, contact dermatitis, or induce vomiting -May exhibit batch-to-batch variation -Depends on the geographical location and season
Animal derived compounds	-Promote wound healing -Anti-inflammatory, antiseptic, and antimicrobial properties -Cell-stimulating properties -Low cost and large availability -Stimulate angiogenesis -Stimulate wound contraction	-May induce pain and contact dermatitis -May exhibit batch-to-batch variation -Depends on the geographical location and season
Living organisms	-Promote wound healing -Anti-inflammatory and antimicrobial properties -Cell-stimulating properties -Low cost and large availability -Stimulate wound debridement -Can be incorporated within dressings	-May promote pain and discomfort -May exhibit batch-to-batch variation -Depends on the geographical location and season
Solutions, creams, and ointments	-Promote wound healing -Not expensive in general -Provide cleaning, disinfection, debridement, and pain-relief -Ease-of-use -Antimicrobial properties -May be embedded within wound dressing	-Short residence time on the wound (require recurrent administrations) -Limited skin regeneration
Autografts	-Reduced rejection -Good adhesion to the wound bed -Skin regeneration for full-thickness skin wounds -Able to replace the lost tissue	-Lengthy hospital stays -Patient's donnor site morbidity -Induce scar formation -Limited availability of donor sites
Allografts	-Prevent wound dehydration and contamination -Skin regeneration for full-thickness skin wounds -Off-the-shelf -Able to replace the lost tissue	-Temporary -Limited availability in donor banks -Risk of immune rejection -Risk of disease transmission

Table 2.3: Skin therapies (2/2)

Technique	Advantages	Disadvantages
Wound dressings	-Provide a protective barrier against microorganisms -Create a warm and moist wound environment, providing favorable conditions for the healing process -More flexible than traditional dressings -Ability to hydrate the wound and remove excess of exudate -Can perform additional functions, such as the release of bioactive agents -Relatively low cost	-Inability to promote the regeneration of lost skin, particularly the dermis -Low adhesion to the wound bed
Bioengineered Skin substitutes	-Template for cell adhesion, proliferation, and differentiation, guiding new tissue formation -Can be used in combination with autograft -Able to replace the lost tissue -May deliver ECM biomolecules to the wound bed, improving the healing process -Prevent fluid loss and protect from contamination -Promote the regeneration of dermis and epidermis, restoring tissue function -Reduce inflammatory responses -Reduce scarring -Reduce hospitalization time and number of required surgical procedures	-Poor adhesion to the wound bed -Difficult handling -Scarring at graft margins -Risk of immune rejection and disease transmission (in the case of allogenic cells) -Poor vascularization -Impossibility to reproduce skin appendages -No large-scale availability -High manufacturing costs and time
Biophysical agents	-Improve the wound healing outcomes	-Cannot be used alone
Bioprinting	-Possibility of precise production of complex and well-organized 3D scaffolds -Possibility of printing biomaterials with embedded cells and biomolecules -Automatic -High resolution, accuracy, and reproducibility -Spatial and concentration control -Enables the fabrication of skin substitute fitting to the anatomical shape of the defect -Precise control over porosity -Able to replace the lost tissue -May have the possibility of vascularization and appendages	-Difficult to replicate cellular and ECM heterogeneity and complex micro-architecture in native skin -Requires the integration of imaging techniques -Requires the use of printable biomaterials exhibiting adequate mechanical and biological properties

2.1.8 Biomaterial and cell requirements for skin applications

Biomaterials and cells have to accomplish certain requirements to be used in skin applications, depending on the application.

2.1.8.1 Biomaterials

Scaffolds play a major role in TE, because the cells attach to them, differentiate, proliferate and exercise their function [110]. The choice of the adequate material for a certain application process, and its performance in a particular application depend on some characteristics, namely [112, 113, 110]:

- **Biocompatibility and cytocompatibility**: Materials must not trigger undesirable responses (local or systemic) by the host, and must contribute in an active and controllable way to the biological function of the construct, being biocompatible for transplantation in the long-term. The bioprinted substitutes that will develop *in vivo* must be receptive to remodeling, facilitating the formation and development of structures driven by physiological mechanisms. The used biomaterials also have to support cell attachment, proliferation and functions. Biocompatibility is not an intrinsic property of a material, but depends on the surrounding biological environment and on the tissue reaction [101];

- **Printability**: Materials used for bioprinting must present properties that facilitate the handling and deposition by the bioprinter. Key parameters include rheological properties, gelation methods and shape fidelity;

- **Biodegradation**: In many applications, it is desirable that the scaffold presents some kind of degradation, but degradation products must be nontoxic. Degradation kinetics is critical, and degradation rates must match the cells' capacity to secrete their own ECM. In other applications the materials may be designed to be stable over time instead of degrading when in contact with biological fluids by enzymatic or hydrolytic (chemical) degradation [73];

- **Structural and mechanical properties**: The choice of the used materials must be based on the desired mechanical properties for the construct, ranging from a soft hydrogel for cell biocompatibility to rigid thermoplastic polymer fibers for strength. Mechanical properties include micro and macrostructure of pores, their size and shape, and mechanical strength. Materials must also present short-term stability in order to keep the initial adequate mechanical properties, ensuring that tissue structures (e.g., networks, channels, pores) do not collapse after printing. Biomaterials must also exhibit suitable contractile or swelling features;

- **Surface chemistry and topography**: In some applications, the surface properties, such as roughness and chemistry, may be important, to elicit specific cell responses.

Currently used biomaterials in skin applications are based on either naturally-derived (e.g., collagen [120, 121], alginate [122, 123], gelatin [124], chitosan [125], hyaluronic acid [126], fibrin [127, 128], decellularized ECM [129], cellulose [130], pectin [131] and silk [132], usually isolated from plant, human, or animal tissue) or synthetic polymers (e.g., polycaprolactone (PCL) [133], poly(lactic-co-glycolic acid) (PLGA) [134], polylactic acid (PLA) [135], polyethyleneglycol (PEG) [136]) [112]. Even though some natural polymers may resemble the ECM in some aspects and exhibit analogous properties, synthetic hydrogels are attractive materials due to the facility to control their physical properties and composition during the synthesis process according to the desired properties, in addition to being more available [110]. Natural polymers generally bring concerns regarding disease transmission, high immunogenicity and weak mechanical properties. Each polymer type presents advantages and disadvantages for a bioprinting type.

2.1.8.2 Cells

Beyond biomaterials, the choice of cell types is also crucial. They must allow expansion into a sufficient number, and their proliferation must be precisely controlled *in vitro* and *in vivo*. The timing of cell proliferation is also important: in the beginning, a high proliferative rate is desired to populate the construct, but then proliferation should stabilize at an adequate rate to reach homeostasis. Cells must be able to maintain their long-term function. It must also be taken into account the cell origin, i.e., autologous, allogenic, or xenogenic, related to rejection by the host immune system and to availability.

Fibroblasts are the main ECM producers, providing a physical support for other cells to adhere, differentiate, proliferate, migrate and perform their biological functions. In addition, they produce biomolecules that are involved in some physiological processes, such as tissue repair and angiogenesis. Fibroblasts, as the main ECM producers, are able to modulate the mechanical and biochemical microenvironment of neighbor cells. Therefore, they have a great potential in orchestrating tissue regeneration, being extensively explored as a cell source for the development of tissue-engineered constructs, namely in skin substitutes [137]. This cell type is easily accessible through skin biopsies and can be used for both autologous and allogenic substitutes. However, donor-to-donor variation leads to differences in host response, but as fibroblasts have immunoregulatory functions, problems regarding rejection in allogenic approaches can be reduced. Another drawback of fibroblasts is the lack of specific markers that clearly identify these cells. For the development of TE constructs using fibroblast-derived ECM, the most presently followed strategy [137] is the *in vitro* culture of isolated fibroblasts over time, allowing them to secrete their own ECM, and afterwards perform decellularization protocols to use the matrix as a physical support for other cells.

Endothelial cells (ECs) are another widely used cells in TE. The genesis of new blood vessels requires not only the assembly of ECs into a tubular structure, but also a support that may be provided by fibroblasts. Fibroblasts appear to be able to modulate angiogenesis. As a result, these cells together with ECs have the potential to promote new blood vessels formation [137].

Mesenchymal stem cells (MSCs) represent the current most promising cell type in the RM, mostly due to their capacity to differentiate in multiple cell lineages under specific culture conditions, including substrate surface physical properties [138, 5].

Other cell, such as stem cells derived from the umbilical cord, have also been used [139].

2.1.8.3 Requirements of skin substitutes

With the aim of closely replicating the structure and properties of native skin, printed skin has to fulfill some functional and compositional features, namely [1]:

- **Cellular composition**: Newly formed skin must replicate the diversified cellular composition of native skin as close as possible. To achieve that, each cell type must be accurately placed at specific 3D positions in the respective layer. The cellular density and the ratio between cell types should also be precisely controlled in order to promote adequate cell-cell interactions;

- **Biochemical composition**: The different skin layers have different cell-ECM ratios and different ECM components, as well as different cell-adhesion sites;

- **Tissue shape and architecture**: Skin thickness may range between 1 and 2.5 mm, depending on the body site. Furthermore, some regions present a well-organized skin, while in others, skin is more randomized. This skin heterogeneity throughout the body needs an accurate control in the components orientations and in the layer thickness;

- **Skin functions**: Materials and cells must be deposited in a way that is possible to recreate the skin function, mainly the barrier function;

- **Appendages and pigmentation**: The production of skin appendages is a huge challenge in the printed skin field;

- **Vascularization**: Vasculature is crucial for the viability and function of all tissues and cells, mainly due to the nutrients and gases supply, and insufficient blood stream may lead to the implanted construct failure. The vascular lattice is also in charge of the transport of metabolites, hormones and antibodies between tissues. The capacity of engineering functional 3D vasculature is pivotal for the success of skin constructs, being one of the major challenges in TE. The use of endothelial cells has successfully been investigated to engineered vascularized tissues, in co-cultures with supporting cells, such as fibroblasts [140].

There are many promising strategies to promote skin wound healing, repair and regeneration, that can be applied directly in the treatment of skin injuries or in the production of functional skin models that can be used in the clinics (implanting the substitute in a patient), or to serve as 3D models to study several mechanisms in the research, or to be used in the pharmaceutic or cosmetics industry, minimizing experiments in animals. As previously mentioned, printed skin constructs

must mimic the native skin in its composition, architecture and functions. To produce such substitutes, several parameters, such as the used technology, the selected material with its concentration and crosslinking methods, chosen cells with their maturation times, must be taken into account according to the final application of the substitute. The production of skin substitutes with functional vascularization and appendages remain the major challenge in the field. Skin bioprinting is a promising approach to build a fully functional skin equivalent presenting adequate vasculature and appendages, by the simultaneously printing of the adequate combination of biomaterials, cells and biomolecules. For that purpose, further research have to be carried out in order to create new strategies, better bioinks and more versatile processing technologies. For each process and application, optimization of parameters and conditions is required, and the outcomes must be evaluated and characterized.

2.2 Polymers used in the project - pectin and chitosan biomaterials

In this section, the polymers used in this project will be addressed: their structure, general properties, characteristics to be used as a dressing material and other properties that may improve wound healing, crosslinking mechanisms, among other aspects. Then, the interactions between polymers will be explored.

2.2.1 Pectin

Pectin (**Figure 2.6**) is an anionic biopolymer extracted from plant cell walls [141, 142, 143], being one of the major structural polysaccharides of higher plant cells.

Pectin is a high molecular weight polymer with a branched structure composed of multiple domains. Pectin is characterized by its branched heteropolysaccharides, which mainly consist of linear chains of partially methyl-esterified (1,4)-alfa-D-galacturonic acid residues in its backbone, interrupted with (1,2)-L-rhamnose residues. Pectin possesses a high content of hydrophilic groups, containing both hydroxyl (OH) and carboxyl (COOH) functional groups [144].

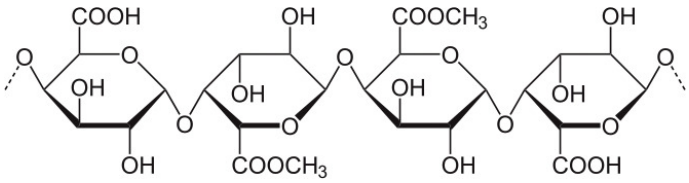

Figure 2.6: Chemical structure of pectin. Image from Kitir *et al.* (2018) [6].

Pectins can be classified according to the degree of esterification (DE) or according to the degree of substitution of D-galacturonic carboxyl groups by methoxyl groups, in high-esterified pectins (DE > 50%) or low-esterified pectins (DE < 50%), presenting distinct properties [7, 142].

Many natural polymers present poor mechanical properties and fast degradation, which limits their applications. As a consequence, crosslinking agents are used to improve mechanical properties. Since pectin is an anionic polymer, it can be ionically crosslinked in the presence of divalent

metal cations, such as calcium ions (Ca^{2+}), in an aqueous medium, forming a hydrogel by simple gelling mechanisms, either internal or external [141, 145]. The affinity of the pectin chain for cations increases with rising charge density along the polymer backbone [141]. Calcium ions tailor the viscoelastic properties through ionic gelation between adjacent groups. The gelation behavior depends on the DE, being the regions of unsubstituted galacturonic acid residues responsible for the crosslinking [141].

Pectin has several potential biomedical applications, such as for drug delivery, wound dressings and tissue engineering [7]. Pectins are a very versatile material, since they are easily adapted to hydrogels, scaffolds, nanoparticles, coatings, and films. It is a very desirable material for wound healing applications due to its non-toxicity, safety, wide availability and low-cost. Furthermore, its simple gelling mechanisms facilitate the production of hydrogels for biomedical applications. Due to its biocompatibility, biodegradability, water-solubility, the ability to form physical hydrogels, and the presence of modifiable functional groups, pectin has been receiving increasing attention in the biomedical field for TERM strategies.

Pectin can also be chemically modified due to its branched structure composed of multiple domains, is provides a great number of target sites for chemical modification [146, 147, 5]. Comparing to other natural polymers, pectin lacks endogenous cell-adhesive sites in its native composition, which provides the possibility of precisely introduce specific moieties onto the otherwise bioinert backbone, allowing the engineering of biofunctionalized macromers for the production of chemically defined hydrogels and to reach the desired properties [146, 147].

In particular, for wound dressings, pectin presents some interesting properties [7, 148, 89]:

- Biocompatibility;
- Biodegradability;
- Bioactivity;
- Non-toxicity;
- Anti-inflammatory, antimicrobial, antiviral, and anti-carcinogenic activity;
- Water-solubility;
- Water absorption capacity;
- Moisture retention ability;
- Adequate gelling properties to form hydrogels;
- Ability to form sponge structures;
- Healing properties;
- Improvement of mechanical properties;
- Safety, low-cost, high availability.

2.2.2 Chitosan

Chitosan (**Figure 2.7**) is a natural and linear polycationic polymer [149, 7, 150, 151, 152, 153, 142, 89]. It is a polysaccharide obtained by enzyme or alkaline N-deacetylation of chitin [149, 7, 150, 151, 89], which is found in the shells of lobsters, shrimps and crabs, being the second most abundant biopolymer in nature, after cellulose [7, 154, 142]. By the process of alkaline hydrolysis of the N–acetyl groups from chitin, (1,4)-beta-D-glucosamine chains are obtained.

Chitosan contains a high content of hydrophilic groups (pectin also has hydrophilic groups), holding a high content of amino (NH_2) and hydroxyl (OH) functional groups [149, 144].

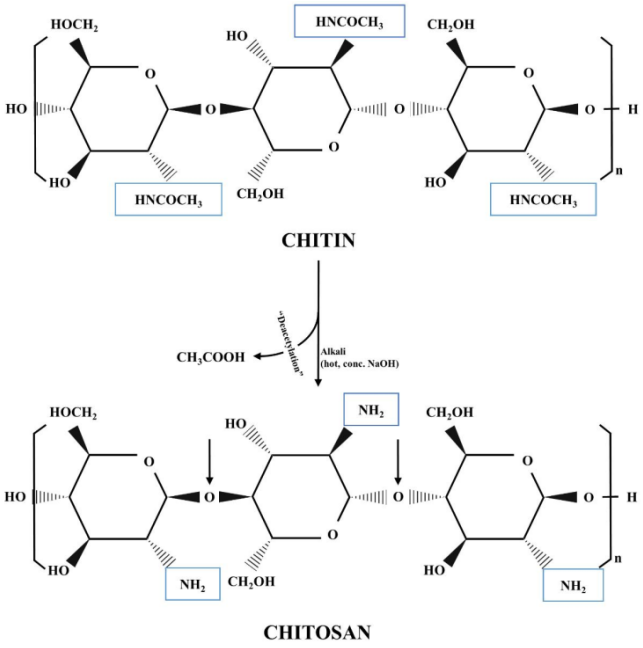

Figure 2.7: Chemical structure of chitin and chitosan, and chitosan production from chitin. Image from Martau *et al.* (2019) [7].

Chitosan is classified according to degree of deacetylation and is composed of two monomers: N-glucosamine and small amounts of N-acetyl glucosamine (acetylated unit) [142].

As a crosslinking agent for chitosan, glutaraldehyde (GA) is usually used due to the fast Schiff base reaction that occurs between the amine groups of chitosan and aldehyde groups of GA [155]. It also can create covalent crosslinking by forming an acetal structures between the CHO groups of GA and OH groups of two polymer strands [156]. Nevertheless, GA is toxic, so its use is not recommended to produce products for biomedical applications. To overcome the problem of toxicity, genipin, a natural crosslinking agent extracted from the gardenia fruit, has been successfully used in the preparation of chitosan-based hydrogels [149, 152, 89]. Genipin has been attracting increasing interest due to its low toxicity comparing to other chemical crosslinking agents, such as GA and formaldehyde. Genipin is able to crosslink macromolecules, such as proteins and polysaccharides, being a well-known crosslinking agent of chitosan, which consists of many amino groups. Genipin reacts with amino groups, being able to improve the mechanical properties of chitosan

[89]. Beyond acting as a natural crosslinker of chitosan, genipin also provides some benefits related with wound healing, such as non-toxicity, biocompatibility, anti-inflammatory properties and improvement of mechanical properties of a material [89]. The degree of crosslinking by genipin is dependent on the available free amino groups available to react with genipin. Chitosan can be also crosslinked by basification, which will be explained in **Section 2.2.3.5**.

As it is a polycation, chitosan has been used to prepare ionically crosslinked hydrogels with anionic polymers, such as pectin and alginate [153, 149].

Due to its outstanding biological properties, chitosan has been used to fabricate various scaffolds and hydrogels that can be applied in many areas of TE, such as skin, nerve, bone, and cartilage [154]. Chitosan can be used to prepare IPN-based hydrogels with other polysaccharides (e.g. for designing drug release systems) [149]. Due to the variety of side groups (amino and hydroxyl) on its backbone, it is an ideal candidate for biofabrication in TE. Being chemically similar to GAGs, an important component in the natural ECM, chitosan is an adequate scaffold material for TE. Chitosan is a widely used material in commercially available wound dressings, being that its properties for these applications were largely studied.

Chitosan, as a weak base, requires a certain amount of acid to convert the glucosamine units into a positively charged form that is soluble in water [7]. Thus, chitosan is not soluble in water, but it needs to be acidified to be soluble in aqueous solutions. As a result, it only can be dissolved in acidic solutions. Given that it also presents poor mechanical strength itself, it is generally combined other materials to enhance mechanical properties (for instance, with anionic polymers in an IPN) [151]. Chitosan is widely used as a gel-forming material to form hydrogels [157].

Chitosan holds a great set of mechanical and biological properties for being used in wound dressings, namely [153, 155, 149, 151, 152, 150, 154, 7, 86, 158, 81, 159, 88, 160, 148, 161, 89]:

- Biocompatible;
- Biodegradable (undergoes enzymatic degradation by lysozyme, human enzyme);
- Biofunctional and bioactive;
- Non-toxic and inducing low immunogenicity;
- Anti-inflammatory, antimicrobial and antibacterial properties;
- Mucoadhesive and cell-adhesive properties;
- It increases the swelling ability of a polyelectrolyte complex;
- Prevents wound dehydration;
- Permeable;
- High capacity of adsorption of dyes, proteins and metal ions;
- Induction of the healing process;

- Ability to form sponge structures;
- Hemostatic activity;
- Stimulates cell proliferation and tissue organization;
- Accelerates wound healing process by the improvement of the functions of macrophages, inflammatory cells and fibroblasts;
- Prevents scar formation;
- Improves mechanical properties.

2.2.3 Interactions between pectin and chitosan

2.2.3.1 Hydrogels

A variety of materials have adequate mechanical and biochemical properties to mimic specific tissues [113]. Hydrogels are soft polymeric 3D crosslinked network structures obtained from a variety of natural or synthetic polymers that swell, absorbing and retaining large amounts of water [1, 162, 73, 156, 150]. Hydrogels are water-insoluble and hydrophilic 3D networks made of physically or chemically crosslinked polymer chains, that exhibit high porosity and permeability to both oxygen and nutrients, which allows the exchange of soluble factors within the gel [162].

Hydrogels can be prepared by physical or chemical crosslinking processes: while permanent or chemical gels are covalently crosslinked networks, reversible or physical ones are held together by molecular forces, as hydrophobic interactions, ionic interactions or hydrogen bonding [149, 150]. Reversible hydrogels can be disintegrated by alterations in the environment, such as temperature, pH and ionic strength [149]. While chemical crosslinking leads to stronger hydrogels, they can have undesirable effects on biomaterials due to the use of cytotoxic crosslinking agents.

Hydrogels are obtained by the hydration of hydrophilic groups from a polymer network in an aqueous environment. A wide variety of hydrophilic polymers (natural, such as polysaccharides, or synthetic) have been used to prepare hydrogels, being that polysaccharides are the most widely used hydrogels in biomedical applications [73]. These polymers contain hydrophilic functional groups such as carboxyl, hydroxyl and amines. By the combination of polymers, IPN composite hydrogels can be prepared [149].

Hydrogels present some interesting properties that turn them into good candidates for many applications in the biomedical field, particularly for skin applications [1, 162], such as wound dressings, tissue engineering scaffolds and drug delivery vehicles, due to the similarity of their properties and the properties of the natural tissues [156]. These properties include high water content, biocompatibility, tissue-like elasticity, high equilibrium water content and the ability to mimic the ECM, providing a physical support for the cells exercise their functions. They are also able to provide moist environment, important to promote the healing process, being excellent candidates for wound dressing application. As previously mentioned when, describing essential

features of dressings, their porosity, permeability to both oxygen and nutrients, low immunogenicity, viscoelastic properties, ability to prevent bacterial invasion, ability to absorb exudates and low adherence to the wound bed are also important features to wound dressings. Hydrogels can also provide a porous and soft matrix for entrapped cells and have the ability to incorporate cells or biomolecules of interest. Some hydrogels can be formed *in situ*, which constitutes a great advantage for transplantation purposes, because injectable materials can be introduced in a minimally invasive way, with lower costs, faster recovery and less discomfort for patients [162, 118].

Although hydrogels are widely explored, there are some TERM applications where hydrogels cannot fulfill on their own. Thus, there is the possibility to carry out some modifications in the hydrogels in order to improve their mechanical and biological properties, since they present tunable properties, i.e., they can be modified to present better or even ideal properties, such as mechanical or rheological properties, cytocompatibility, biodegradability and bioactivity, allowing control over stability, permeability, and biodegradability rate [113]. Hydrogels can be incorporated with reinforcing structures (creating hybrid hydrogels), or be chemically or physically modified. The incorporation of several types of structures can be achieved through the integration of secondary polymer networks [163], embedding of micro or nanoparticles or even integration of fibrous structures. The reinforcement to be used will depend on the biological and mechanical desired outcomes. They can enhance mechanical properties (such as strength or elasticity, depending on the application), guide organization of the cells through specific patterns and orientations and stimulate several responses on hydrogels, by the incorporation of structures with thermal or electrical properties, for instance [164]. In order to recreate some characteristics of the native ECM and improve their biomimetic properties, hydrogels may be biofunctionalized with specific cell-instructive moieties. Biophysical properties can also be tuned to mimic the native ECM mechanical properties [162]. Cell-instructive hydrogels are important agents in TE owing to their resemblance to the natural ECM, being able to influence the behaviour of embedded cells.

2.2.3.2 Polyelectrolyte complexes

A polyelectrolyte complex (PEC) is formed by ionic interactions between polyanionic and polycationic polymer, presenting unique properties which differ from those of the initial components. The combination of polymers in a PEC is a common strategy to enhance specific properties of a single material, such as mechanical or biological [142]. PECs are 3D networks prepared by noncovalent bindings between the oppositely charged polyions. The physical nature of the interactions between the cationic and anionic groups is reversible and depends on the properties of the reacting polymers, such as degree of ionization, ionic strength, polymer conformation, and molecular weight [89]. During the formation of a PEC, for instance, the amino groups of the polycationic polymer can interact with the carboxyl groups of polyanionic polymers mainly by electrostatic forces, but also by other types of interactions, such as hydrophobic attraction, transfer forces, van der Waals forces, Coulomb forces, and hydrogen bounding, can also occur between the oppositely charged polymers [142, 88, 161]. The state (gel, collapsed system, phase separated system) and

stability of PEC systems depends on temperature, ionic strength, pH, and charge density, counterion concentration and type, among other environmental conditions [141, 142].

PECs present better mechanical stability and structural strength than the constituent polymers alone. Also, PECs change the tendency of swelling of the materials [158, 81]. PECs offer the advantages of both of the combined materials and can be tailored for several biomedical applications, such as scaffolds, membranes, wound dressings, coatings, packaging, microcapsules and fieres, having many and varied applications [158, 81, 89]. PECs formed by blending oppositely charged biopolymers have been receiving much interest as wound healing materials due to their unique biological and physicochemical properties [88, 161]. Biopolymers, including proteins, nucleic acids and polysaccharides, are often polyelectrolytes, and these polymers have important applications as coatings, gelling agents, and thickeners [141].

2.2.3.3 Polyelectrolyte complexes between pectin and chitosan

Chitosan, due to the presence of primary amino groups, can form PECs in the form of membranes, hydrogels, particles and beads, with anionic polymers, such as pectin, alginate, carboxymethyl cellulose and collagen [88, 161]. Chitosan (cationic polymer) and pectin (anionic polymer) form a PEC in the presence of each other by ionic interactions between the amino groups of chitosan and the carboxyl groups of pectin, as they have opposite charges.

Polysaccharides of opposite charge, such as pectin and chitosan, present an attractive interaction, being able to establish very strong intermolecular interactions, being able to form a PEC together [153, 142]. These strong interactions are due to the existence of polar functional groups in the polysaccharide structures, which also leads to a highly ordered orientation of the polymer chains and great structural stability since both pectin and chitosan present high hydrophilicity [142], and also resulting in a high amount of bound water in the complex [144]. The electrostatic attractions between the ionized anionic carboxyl acid groups (COO^-) of pectin and the ionized cationic amino groups of chitosan (NH_3^+), formed in acidic environment, are the main interactions in the formation of the chitosan/pectin PEC, forming ionic bonds between them [153, 142, 150, 158, 165, 166]. Also, the NH_2 groups in chitosan are able to form hydrogen bonds with COOH and OH groups of pectin [88].

Chitosan-pectin complexes present ionic interactions between negatively charged pectin and positively charged chitosan, also exhibiting pH-sensitive swelling. In an acidic medium, pectin is neutralized, and free positive charges (NH_3^+) start appearing within the gel, while in a basic medium, chitosan is neutralized, with the emergence of free negative charges (COO^-). The reciprocal repulsion between opposite charges and the entry of water, together with counterions to neutralize these charges, causes swelling [153].

Pectin-chitosan PECs are usually prepared by blending of chitosan and pectin solutions. Amino groups of chitosan are protonated into NH_3^+ groups at pH 5–6 (as already described, chitosan has to be dissolved in an acidic environment). After mixing these solutions, the PEC is formed due to the strong electrostatic interactions between both polymers [161].

There has been an increasing interest in the chitosan–alginate and chitosan-pectin PEC systems to be used in TE [158], due to their structural stability. Chitosan–alginate PEC and chitosan-pectin PEC membranes can be prepared as a wound dressing by gradually mixing solutions of both networks [158].

2.2.3.4 Interpenetrating polymer networks (IPN)

IPNs are the combination of two or more interlaced crosslinked polymer networks in order to form a network. IPNs are composed of two crosslinked networks that are topologically entangled and cannot be separated without disrupting existing chemical bonds [156, 8, 149, 167]. Covalent crosslinking leads to the formation of IPNs with a permanent network structure, since irreversible chemical links are formed. The different polymer chains are not covalently bonded to each other or by any chemical bond [149]. Since both networks in an IPN structure are independently crosslinked and not chemically bound, it is possible to tune the hydrogel properties by independently varying the concentration of each component. The formation of an IPN structure ensures that components remain entangled and there is a resistance to phase separation. IPNs not only preserve the desired features of each network structure, but also enhance the mechanical properties and stability of the crosslinked networks [154].

When IPNs consist of hydrophilic polymers, they swell and form IPN hydrogels [154] by one of the 3 routes presented in (**Figure 2.8**). IPN hydrogels are composed of two or more polymers that are either synthesized and/or crosslinked to each other *via* chemical or physical methods [143]. At least one of the polymers is synthesized and/or crosslinked within the immediate presence of the other to provide a 3D network structure [156, 149, 167]. If two different polymers are independently crosslinked, permeating with each other, the IPN is called full-IPN. On the other hand, if only one component of the assembly is crosslinked, leaving the other one in the linear form, it is called semi-IPN. Unlike PECs, in which both polymer chains are not crosslinked, in IPNs at least one of them is crosslinked.

IPNs can be classified based on their synthesis process. IPNs can be synthesized from monomers or linear polymers simultaneously or sequentially (**Figure 2.8**) [8, 149, 167].

- **Simultaneous IPN:** Both networks that will compose the IPN are polymerized concurrently. The precursors of both networks are mixed and the two networks are produced at the same time by independent and noninterfering routs;

- **Sequential IPN:** The second polymeric component network is polymerized only after the complete polymerization of the first component network. Typically, it is performed by swelling of a single-network hydrogel into a solution containing a mixture of the monomer of the second network, an initiator, an activator and a crosslinker;

- **Sequential IPN by selective crosslinking:** A selective crosslinking process is performed in order to crosslink one network before the other one, by ultraviolet radiation for instance.

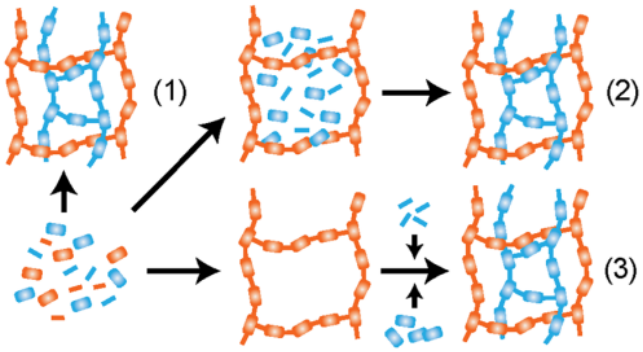

Figure 2.8: Processes for IPN formation. (1) Simultaneous crosslinking. (2) Sequential crosslinking by selective crosslinking. (3) Sequential crosslinking by adding a linear polymer of monomers to an existing single polymer hydrogel. Image from Crosby *et al.* (2020) [8].

IPNs present some interesting properties, including improved mechanical properties comparing to the individual polymer networks that compose it [149, 143]. Single network hydrogels often present weaker mechanical properties and slower response at swelling [149]. IPNs do not dissolve when immersed in a solvent and are resistant to flow and creep. IPN hydrogels can provide an adhesive ECM-like 3D microenvironment to cells, while possessing the mechanical integrity to withstand physiological forces. Usually the former polymer offers good strength, while the latter polymer offers good impact resistance [168].

The polymer types, concentrations, network preparation and crosslinking method are the main factors that influence the network properties [169].

IPN hydrogels can be synthesized from natural and synthetic polymers, synthetic polymers, and combinations thereof. Natural or synthetic polymers containing hydrophilic groups (such as COOH, OH or amines) can be used to produce IPN hydrogels. Usually, biological derived polymers undergo physical crosslinking mechanisms to enhance their mechanical properties. On the other hand, synthetic polymers, such as PEG, PCL or PLA are engineered to display adequate mechanical properties and to incorporate the adequate motifs [8]. Synthetic polymers are usually more resistant to deformation and less degradable than the biological ones, so their use into biological hydrogels is to fine-tune the properties of the hydrogel, improving the strength and stability [8].

2.2.3.5 IPNs between pectin and chitosan

In order to form a pectin-chitosan IPN hydrogel from a pectin-chitosan PEC, some crosslinking agents may be used in order to crosslink one or both polymers that constitute the pectin-chitosan PEC, namely [89]:

- **$CaCl_2$:** This crosslinking agent is able to crosslink pectin carboxyl groups that were not bound to the chitosan amino groups (due to Ca^{2+}, as already previously described, being the most used crosslinking agent for pectin;

- **NaOH:** NaOH is widely used on the crosslinking of chitosan-pectin PECs, due to several aspects. Chitosan acts as an effective crosslinker of concentrated pectin solutions at pH 5.5, being that the efficiency of this process depends on pH, concentration of crosslinker, and the charge density [141]. Also, chitosan itself can be crosslinked by neutralization in dilute NaOH solution to form a hydrophobic network. This process, called basification, reduces the electrostatic repulsion of protonated NH_3^+ (formed in acidic medium, where chitosan was dissolved, when the free amino groups (NH_2) become protonated into NH_3^+), reducing the electrostatic repulsion between chitosan molecules, which encourages the otherwise positively charged chitosan to crosslink *via* hydrophobic and hydrogen bonds. This chitosan network can entangle and interpenetrate with another crosslinked polymer network that may be present in the solution, blending them, forming an IPN structure [8, 149, 154]. Also, this pH value produces the highest product yield in the formation of a PEC between pectin and chitosan [141];

- **Genipin:** This is a commonly used crosslinking agent to chitosan (as already addressed). It is a chemical crosslinker and has been widely used to form IPNs [152, 170].

- **Carbodiimide:** This is an amide-type crosslinker that the ability to crosslink directly the polypeptide chains involving the activation of the carboxylic acid groups. It can crosslink both pectin and chitosan, reacting with their amine groups, and is a chemical crosslinker [152, 171, 170, 172];

- **Glutaraldehyde:** This is a widely used crosslinking agent for chitosan (as already). It is a chemical crosslinker [157, 170].

There is the possibility to crosslink only one polymer or both, forming a semi- or a full-IPN, and they may crosslink at the same time or sequentially. The gelation behavior depends on the pectin DE. Gel stiffness increases with increasing concentration of crosslinker [141].

Chemical crosslinkers are widely used to improve the mechanical properties of hydrogels, but the remaining unreacted chemical crosslinking agents, such as GA and toxic products may turn these hydrogels dangerous for TE applications. Genipin is also used, but it possesses some genotoxicity. So, in order to avoid the use of this type of crosslinking agents, the processes must be optimized in order to not need them, using alternatives to them. Physically crosslinked hydrogels are more attractive for biomedical applications because their preparation avoid chemical crosslinkers, that are typically toxic [148]. Methods for chemical crosslinking include photopolymerization, covalent crosslinking *via* linkers, radical polymerization and enzymatic reactions. On the other hand, physical crosslinking creates a non-permanent network with physical interactions by electrostatic interactions, hydrogen bonds, crystal formation or physical entanglements, being that physically crosslinked hydrogels can be formed *via* crystallization, graft copolymers, ion interactions, and stereo complex formation [150].

Chapter 3

Motivation and specific aims

In this Chapter, the aim, context and goals of this project will be described.

The main aim of this project is to develop and characterize a novel wound dressing based on pectin and chitosan without using chemical crosslinking agents, exhibiting adequate characteristics that would support their subsequent biological evaluation.

The motivation underlying this project was to avoid the chemical modification of polymers and, therefore, explore the unique characteristics of pectin and chitosan in their native state for the design of a novel wound dressing. Taking advantage of the unique properties of chitosan and pectin, namely the antimicrobial properties of chitosan and the ionic gelation of pectin, in addition to their complementary gelation was the rational for the formation of a polyelectrolyte complex (PEC). Then, if needed, complement the mechanical properties producing an IPN hydrogel, crosslinking one or both polymer chains. Finally, freeze-dry the hydrogels to obtain sponges, testing them.

In a first stage, the project focused on the systematic screening of some parameters involved in PEC formation including polymer concentrations as well as crosslinker content and treatment time. This is essential to understand the influence of each polymer and crosslinking mechanism in key characteristics of wound dressings such as the degradation, water-absorption and mechanical properties. Therefore, a methodology for the production of the dressings was established. Then, the samples were tested by quantitative assays, namely regarding mechanical properties, absorption of water, evaporative water loss, water vapor transmission rate and in vitro degradation.

The specific goal of this project is to produce a wound dressing that is expected to fulfill key requirements to be used on wound care, such as:

- **Biocompatibility:** The produced dressings must be cytocompatible in the presence of living cells, namely skin cells;
- **Protection:** The dressing must conform and adhere to the wound bed in order to prevent the entrance of microorganisms and dehydration, maintaining a moist environment;
- **Adequate mechanical properties:** The dressing must be flexible, in order to fit the lesion site, adhering to the wound bed, and robust, in order to not break easily;

- **Ease of handling:** The dressing must be easily manipulated by the health care provider and can be cut to fit the lesion shape;
- **Water absorption ability:** The dressing must be able to remove the excess of exudate without drying the wound bed;
- **Allow gaseous exchanges:** The dressings must be permeable to gases;
- **Biological properties:** In the future, some studies may be carried out in order to find out if these dressings can somehow promote cell adhesion and proliferation, wound healing, repair and regeneration.

Chapter 4

Materials and Methods

4.1 Materials and Reagents

4.1.1 Polymers

Low molecular weight chitosan was acquired from Sigma-Aldrich (448869), and used as received. Raw pectin was purified to remove contaminants, as described in **Section 4.2.2**.

4.1.2 Solutions and Reagents

Acetic acid solution (>99%), from Fluka Chemical®, calcium chloride dihydrate powder (99+%) from Acros Organics®, sodium hydroxide pellets (molecular weight of 40 g/mol) from Fisher Scientific®, and sodium chloride powder were used to produce the solutions described in **Section 4.2.1**.

Deionized water (ddH_2O) was also used. It is a water that has had almost of its mineral ions removed, such as cations or anions, and present a controlled quantity of salts. This water is usually used to wash the samples in order to remove non-reacted substances, such as excesses of NaOH.

A PBS (Phosphate-buffered saline) solution was used. It is a buffer water-based solution containing mainly disodium hydrogen phosphate and sodium chloride. The ion concentrations and osmolarity match those of the human body, so it is usually used in biological research to simulate the body fluids. It has phosphates, that may swap with calcium ions.

DMEM (Eagle's minimal essential medium) was also used. It is a culture medium that provides nutrition to cells and is used in biological evaluation of cell-material interactions. DMEM contains phenol red, which is a pH indicator. At physiological pH, the color of the medium is pink-red. When it is acidic, the color turns into a yellow-orange, and when it is basic, it turns purple.

Ultrapure water (MiliQ®) was also used.

4.1.3 Equipment

A texture analyser TA.XT plus (Stable Micro Systems, Godalming, UK) was used to perform mechanical tests. A freeze-drier (-50ºC) was used in order to perform freeze-drying processes carried out during the project. An incubator was used to incubate samples. A -20ºC freezer was used to freeze samples. An analytical balance was used for weighting. These equipment were all provided by i3S.

4.1.4 Lab materials

In order to carry out this work, the currently used lab materials, such as pipettes, fluid viscous pipettes, Eppendorf tubes or well-plates, were provided by i3S.

4.2 Methods

4.2.1 Production of stock solutions

A 1% (v/v) acetic acid solution was prepared, by adding 1 mL of acetic acid into 99 mL of water (MiliQ®; which will always be used, from here on), in order to prepare a stock of this solution. This solution at this concentration is adequate to dissolve chitosan powder, according to the literature [144, 81, 159, 165, 160, 161, 89].

A 0.9% (w/v) NaCl solution was prepared, by dissolving 0.9 g of NaCl in 100 mL of water, in order to prepare a stock of this solution. This solution is used to dissolve pectin.

Dihydrate $CaCl_2$ powder was used to prepare the dihydrate $CaCl_2$ solutions at the desired concentrations, in order to crosslink pectin. Stocks of 1.25, 2.50 and 5.00% (w/v) were prepared by dissolving $CaCl_2$ in water, and were vortexed to dissolve. According to the literature, $CaCl_2$ is the most used agent to crosslink polymers like alginate and pectin, and its concentration usually ranges between 1% to 5% [81, 173, 174]. The tested concentrations (1.25, 2.5 and 5%) were selected based on the literature to study its influence on the material properties.

Sodium hydroxide (NaOH) pellets were used to prepare the NaOH solutions at the desired concentrations, in order to crosslink chitosan. Stocks of 0.1, 0.5 and 1.0 M were prepared by dissolving NaOH pellets in water. These NaOH solutions were used in the basification step, as the usual concentrations referred in the literature are between 0.1 and 1.0 M [142, 8, 154, 81, 88, 161, 89]. The tested concentrations (0.1, 0.5 and 1.0 M) were decided in order to have a proportionality around this range of values.

4.2.2 Pectin purification

Biopolymers extracted from the nature often contain contaminants that have to be removed for further use in the human body [145]. The first step needed is to purify pectin to be used, since there are no purified pectins available on the market [145]. This purification process is simple, low-cost, and effective, avoiding high amplitude temperature, pH shifts and the use of organic detergents

or solvents. This step is needed in order to remove the impurities of pectin, to have the polymer ready for further use. The purification process also influences the gelation and consequently the viscoelastic properties of pectin. The removal of impurities allows a better accessibility to COO^- of pectin from the Ca^{2+} ions, leading to a more efficient crosslinking [145]. Thus, a purification process was performed in pectin, adapting the protocol described by Neves *et al.* (2015) [145], in order to turn raw pectin into purified pectin. This process reduces the levels of the most common contaminants in plant polysaccharides [145].

A 1% (w/v) pectin solution was prepared by dissolution in water, by dissolving 7 g of pectin in 700 mL of water, under constant stirring. After complete dissolution, pH was adjusted to 6 with 5 M NaOH under constant stirring, measuring the pH with a pHmeter (note that this step has to be carefully performed, drop-by-drop and letting the pH value stabilize, because the pH value must not pass 6, otherwise it must be discarded). Afterwards, successive filtrations were performed using 0.45 μm and 0.22 μm cellulose ester filter membranes (millipore®), using a vacuum pump by suction filtration. Then, 7 g of activated charcoal were added to the filtered solution (1 g of activated charcoal by each gram of pectin used). The addition of this component is an additional purification step, because activated charcoal has the ability to adsorb different types of compounds, so its usage, complementing with filter membranes and ultracentrifugation, leads to a decrease of the major contaminants, without altering the physicochemical properties of pectin. The suspension was stirred for 1 h at room temperature. Afterwards, the suspension containing the purified pectin and activated charcoal was divided into falcon tubes with the same weight, which were then centrifuged. The suspension was centrifuged for 1 h at 22,000 rpm at room temperature. After centrifugation, the pectin was filtered, frozen at -20ºC, freeze-dried for 2 days, and stored at -20ºC until further use. The result is a solid pectin, with a sponge-like structure.

4.2.3 Qualitative optimization of wound dressing formation

In order to optimize the parameters to implement an adequate protocol to produce consistent hydrogels that can be freeze-dried into stable wound dressings, qualitative optimization tests were performed.

This optimization process was qualitative, in order to determine suitable parameters and procedures to use in order to obtain gels with the better properties and that form stable sponges after freeze-drying. All the possible variables were mapped, in order to understand the best combinations to obtain a gel with better properties that may after be freeze-dried, by qualitatively evaluating the gel's properties, including stiffness, ease of handling, integer structure, cohesion, consistency, stability/dissolution, visual aspect and homogeneity.

In most of the studies available in literature [81, 173, 88, 159, 89], dressings were made using chitosan and alginate, instead of pectin, although alginate is chemically similar to pectin and, thus, some variables could be extrapolated and used as reference. Furthermore, some protocols with pectin confirm the similarity of conditions to use [142, 148, 161]. Nevertheless, some features of the materials, such as molecular weight, may vary. Therefore, an optimization was very important, to find the better parameters in an experimental way for the available materials.

In order to screen the concentrations and parameters to obtain a gel with good consistency and integrity, the following variables were qualitatively tested:

- Determination of the dissolution limit of each polymer;
- Determination of the best pectin-chitosan ratio;
- Determination of the polymer concentration to form a consistent and homogeneous hydrogel;
- Determination of the optimal crosslinking methods for hydrogel formation;
- Determination of the concentration of each crosslinking agent;
- Assessment of the efficacy of crosslinking by stirring or diffusion;
- Determination of the crosslinking time;
- Determination of the best step in the fabrication sequence to perform freeze-drying;
- Qualitative comparison of the degradation behavior of the samples before and after freeze-drying.

These variables are explained in the following Subsubsections. The results and discussion for each variable are described in **Section 5.2**.

4.2.3.1 Determination of the dissolution limit of each polymer

The maximum concentration of each polymer (pectin and chitosan) that can be completely dissolved was tested. Although these values were already described in the literature, they depend on the molecular weight of the specific polymer, and hence this parameter was optimized to the used pectin and chitosan. The solubility was addressed in a qualitative way by observing the homogeneity, opacity, presence of solid particles and by verifying if it was able to be pipetted with a pipette specific for viscous fluids. In order to determine the limit dissolution concentration of each polymer, the following procedure was followed: chitosan and pectin stocks at different concentrations were prepared - each polymer was dissolved in an Eppendorf tube, using a vortex, in the respective solution (chitosan in acetic acid and pectin in a NaCl solution), at the desired concentrations. The tested concentrations for both polymers were 2, 4, 6, 8 and 10%. For example, to produce a 4% chitosan solution with a volume of 1000 μL, 40 mg of chitosan is dissolved in 1000 μL of the acetic acid solution. The maximum limit can be determined by assessing if the solution was heterogeneous and could not turn into homogeneous, if it was impossible to pipette with a pipette for viscous fluids, and if it presented solid particles that could not be dissolved.

4.2.3.2 Determination of the best pectin-chitosan ratio

Different pectin-chitosan ratios were studied, using only the mixture between them, being treated with either $CaCl_2$, NaOH, or with both agents in different sequences. These combinations were tested in 3 different ratios: 1:3, 3:1, and 2:2, evaluating which pectin-chitosan ratio produced a gel with better consistency.

4.2.3.3 Determination of the polymer concentration to form a consistent and homogeneous hydrogel

In order to address which were the best concentrations of each polymer on the mixture solution in order to produce a gel with consistency and homogeneity, the PEC formed between both polymers was evaluated with different concentrations. Considering that the maximum limit of dissolution of chitosan was 6% (as explained in **Section 5.2.1**) and the best ratio between pectin and chitosan is 1:1 (as explained in **Section 5.2.2**), the maximum limit in which each polymer will be dissolved is 6%. Also, it is important to consider that if both polymers are dissolved at 6% and then mixed to form a PEC, this PEC solution present 3% of each polymer.

Usually, studies that develop this type of dressings, pectin and chitosan are dissolved at concentrations between 0.5 and 4.0% (w/v) and then mixed [142, 158, 173, 81, 159, 88, 161, 89]. However, some optimization must be done in this step, because this parameter strongly depends on the molecular weight of the polymers.

Therefore, in order to evaluate the combination of the two polymers, pectin and chitosan were dissolved at 2, 4 and 6%, and then mixed at 1:1 ratio, forming a PEC solution with 1, 2 and 3% of each polymer, respectively. The miscibility of the two polymers at these concentrations, homogeneity of the mixture and the consistency of the formed gels were evaluated, in order to determine the ideal concentration of the polymers for gel formation.

In order to determine the ideal concentrations of each polymer to form a gel, the following procedure was followed: both polymers were dissolved, producing stocks of 2, 4, and 6% of each one, and mixed in a 1:1 ratio, combining both polymers dissolved at the same concentrations, obtaining a PEC with 1, 2 and 3% of each polymer. The mixture was homogenized and centrifuged to remove trapped air bubbles [159]. The reaction between the two polymers was almost instantaneous. Then, each combination was placed in a mold and treated with $CaCl_2$ and NaOH to achieve crosslinking (as it will be described in **Section 4.2.3.4**). Then, the discs were removed from the mold and the properties were evaluated in a qualitative way.

4.2.3.4 Determination of the optimal crosslinking methods for hydrogel formation

As already described in **Section 2.2.3.5**, $CaCl_2$ and NaOH are the most commonly used crosslinking methods applied to pectin-chitosan PECs to form a semi- or full-IPN by the crosslinking of one or both polymers. Most of the works involve this type of dressings use $CaCl_2$ and NaOH as crosslinking mechanisms, but its sequence may change [81, 173, 159, 88, 148, 161]. So, this parameter still has to be further studied.

In order to better assess each step, with the treatment of both crosslinking agents in the PEC, some tests were performed. These tests made possible to understand what happened in each step and in each reaction (namely if it dissolved, crosslinked, precipitated, etc.), and to understand which crosslinking methods are better in producing a consistent gel. These tests were carried out in order to understand the influence of $CaCl_2$ and NaOH in pectin, chitosan and in the pectin-chitosan PEC, evaluating also the best sequence of treatments.

In order to perform these tests, pectin and chitosan were both dissolved at 4% in the respective solution, forming a PEC with 2% of each polymer. This concentration was used because: i) it was defined as the minimum concentration that form a hydrogel (as explored in **Section 5.2.3**); ii) this step was only performed to understand the reactions; and iii) in this way less reagents were spent. Droplets of each one of the following combinations were tested in each step. The duration of treatment of each crosslinker was 5 min (the external crosslinking reaction was fast in such small droplets). The visual aspect, consistency and stiffness were qualitatively evaluated. $CaCl_2$ at 2.5% (w/v) and NaOH at 0.5 M were used. These combinations were:

- Pectin in $CaCl_2$ and then in NaOH;
- Chitosan in $CaCl_2$ and then in NaOH;
- Pectin in NaOH and then in $CaCl_2$;
- Chitosan in NaOH and then in $CaCl_2$;
- All the previous samples in distilled water (after gelification, if they did not dissolve);
- Pectin in distilled water;
- Chitosan in distilled water;
- Pectin-chitosan PEC in distilled water;
- Pectin-chitosan PEC in $CaCl_2$ and then in NaOH;
- Pectin-chitosan PEC in NaOH and then in $CaCl_2$;
- Pectin-chitosan PEC in $CaCl_2$ and NaOH at the same time.

4.2.3.5 Determination of the concentration of each crosslinking agent

In order to test the influence of different concentrations of the crosslinkers, $CaCl_2$ and NaOH, a PEC of 2% of each polymer was treated with $CaCl_2$ at 1.25, 2.50 and 5.00% and NaOH at 0.1, 0.5 and 1.0 M, in all possible combinations. According to the results described in **Section 5.2.4**, the sequence used was $CaCl_2$ followed by NaOH. The consistency, structural integrity and visual aspect of the formed gels were qualitatively evaluated.

4.2.3.6 Assessment of the efficacy of crosslinking by stirring or diffusion

In order to evaluate whether crosslinking under stirring or via diffusion promotes the formation of a more homogeneous gel network, a PEC stock was prepared, and the two methods were tested. Crosslinkers were added during stirring or by immersion of the PEC in the crosslinkers.

4.2.3.7 Determination of the crosslinking time

A pectin-chitosan PEC stock of 2% of each polymer was produced. Samples were treated with $CaCl_2$ at 2.5% and NaOH at 0.5 M, in different times for each treatment, namely: 3, 10, 30 and 45 min. These times are the usually studied for crosslinking methods and basification step for this type of dressings [142, 8, 154].

4.2.3.8 Determination of the best step in the fabrication sequence to perform freeze-drying

After defining the crosslinking ($CaCl_2$ followed by NaOH), it was evaluated in which step the freeze-drying process would be carried out in order to produce more stable sponges: i) before any crosslinking treatment, (ii) after treatment with $CaCl_2$ but before basification with NaOH, or (iii) at the end of both crosslinking agents, with or without washing steps. The sponges resulting from freeze-drying were treated in order that all the tested combinations were equally treated with both crosslinking mechanisms, only differing on which step the freeze-drying was performed. After all treatments, the samples were immersed in different solutions in order to observe how they behave in these solutions, to determine their stability and to assess if they degrade or dissolve.

Freeze-drying is basically a process in which a solvent is removed from a sample, by a sublimation process, at low temperature and pressure conditions. Before freeze-drying, samples must be frozen. Then, the product is subjected to a deep vacuum, well below the triple point of water. Finally, a drying process is carried out, where heat energy is added to the product causing the ice to sublime. In the case of this work, the samples were always frozen at -20ºC, and the freeze-drying process was performed at 0.200 mbar and -50ºC.

For this test, pectin and chitosan were dissolved at 4% in the respective solutions, and then mixed in a 1:1 ratio, forming a stock of the pectin-chitosan PEC. Then, different combinations were tested in order to understand in which step the freeze-drying step was more efficient. The duration of treatments (30 min) and 2.5% of $CaCl_2$ and 0.5 M of NaOH were used. The 5 formulations to test are described below, and 3 samples were tested for each condition:

- (1) PEC formation $\rightarrow$ freeze-drying $\rightarrow$ $CaCl_2$ $\rightarrow$ NaOH $\rightarrow$ washing with distilled water $\rightarrow$ incubation in different solutions;
- (2) PEC formation $\rightarrow$ $CaCl_2$ $\rightarrow$ freeze-drying $\rightarrow$ NaOH $\rightarrow$ washing with distilled water $\rightarrow$ incubation in different solutions;
- (3) PEC formation $\rightarrow$ $CaCl_2$ $\rightarrow$ NaOH $\rightarrow$ freeze-drying $\rightarrow$ incubation in different solutions;

- (4) PEC formation → $CaCl_2$ → NaOH → freeze-drying → washing with distilled water → incubation in different solutions;

- (5) PEC formation → $CaCl_2$ → NaOH → washing with distilled water → freeze-drying → incubation in different solutions;

The samples, in the gel form, were freeze-dried at -50ºC and 0.200 mbar, during 24 h (time in which the samples did not present any remnant of water) [81, 173, 159, 88, 161]. After freeze-drying, incubation was performed in different solutions (water, PBS, and DMEM) at 37ºC for 24 h, in order to observe their behavior (stability or degradation). The washing step (for the samples that were washed) was performed with ultrapure water for 2 h, with water changes every 30 min.

4.2.3.9 Qualitative comparison of the degradation behavior of the samples before and after freeze-drying

In order to assess if the samples have the same behavior before and after freeze-drying, i.e., in form of hydrogel or sponge, samples were prepared in different compositions (pectin-chitosan PEC, PEC treated with $CaCl_2$, and PEC treated with $CaCl_2$ and NaOH), being that 3 samples of each formulation were freeze-dried and the other 3 did not. As already described, a pectin-chitosan PEC is formed by the blending of both polymers, that interact by electrostatic interactions. An IPN is formed when at least one of the polymers is crosslinked (by $CaCl_2$ or NaOH in this case). Then, the samples were immersed in different solutions (distilled water, PBS and DMEM), and their stability was evaluated. Therefore, this behavior was compared. This procedure is described in **Figure 4.1**.

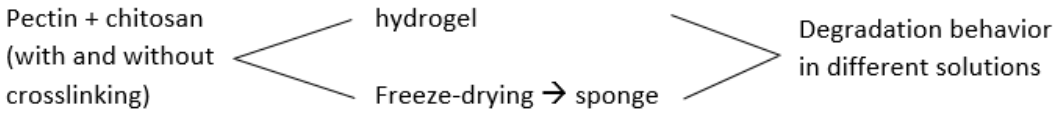

Figure 4.1: Comparison of the degradation behavior oh hydrogels and freeze-dried sponges.

Until now, the focus of the experimental work was in the optimization of the fabrication process (**Figure 4.2** represents the tests carried out during this qualitative optimization), in order to select parameters to achieve the best strategy for the production of wound dressings. With so many different parameters to evaluate, qualitative tests were performed to reduce the number of parameters to evaluate in quantitative tests. After defining all parameters, quantitative tests need to be carried out to quantify key properties and select the optimal formulations.

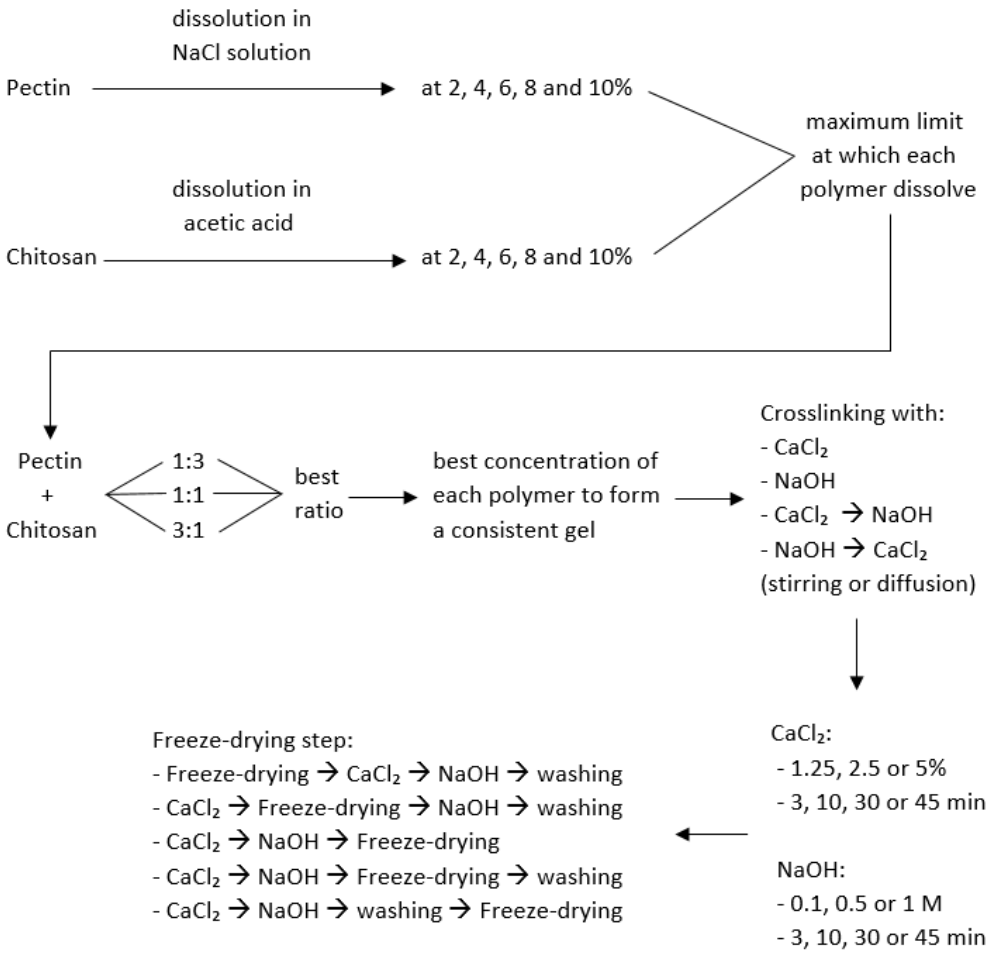

Figure 4.2: Methods for the qualitative optimization of the steps involved in the fabrication of wound dressings.

4.2.4 Quantitative characterization of wound dressings

4.2.4.1 Mechanical tests

In order to compare the different combinations of parameters, and the influence of each variable in the mechanical properties of the dressings, mechanical tests were performed on the samples. Usually, the main disadvantage of sponges in wound healing process is lack of stability and weak mechanical resistance [89]. Thus, the stability and mechanical strength must be tested.

Dressings are applied to the skin, thus being constantly subjected to compressive forces, among other [175]. The samples were mechanically characterized by compression tests at room temperature using a software-controlled Texture Analyser (TA.XT plus texture analyzer; Stable Micro Systems, Godalming, UK), with a load cell of 5 Kg, force resolution of 0.01 g and distance resolution of 0.001 mm[1].

[1] https://www.stablemicrosystems.com/TAXTplus.html

First of all, a test was carried out in order to optimize the parameters to use in order to create a well-defined protocol, namely thickness and surface area of the samples to test, probe to use, and parameters to use in the software. This optimization was performed evaluating if the device presented sensitivity enough. After this optimization process, and according to values described in the literature for similar tests, the adequate values were defined [154, 161, 89, 175, 176].

To prepare the samples, pectin and chitosan were weighted and dissolved in the respective solution at the desired concentrations. The dissolved polymers were mixed at a 1:1 ratio and homogenized. The stock of the mixture was distributed by molds, in order to normalize thickness and surface area. The samples were treated with the adequate concentrations and times of $CaCl_2$ and NaOH (100 μL of each crosslinker in each sample, covering it), in the sequence previously decided, and then washed by 2 h with water, that was changed every 30 min. Then, the samples were frozen at -20ºC overnight. Afterwards, they were freeze-dried for 24 h and then incubated at 37ºC in 500 μL of culture media for 24 h to hydrate [141]. Dressings are used in skin wounds, which are a moist environment and, thus, the dressing needs to be resistant in a wet state. Hence, testing samples in hydrated samples is imperative. Furthermore, in the clinical point of view, wound dressings must be manipulated in a wet state without breaking up.

After incubation, the excess of media from the surface was gently removed with filter paper, and the diameter and thickness of each sample were measured with a digital caliper (average of 5 measurements) to calculate stress and strain [166]. Finally, compression tests were performed. Five samples of each testing group were produced, and the average value of the measurements was recorded for each formulation [154, 89].

According to what was defined in **Subsubsection 5.2.10**, differences in the relative polymer concentration in the PEC (2% and 3%), $CaCl_2$ concentration (1.25, 2.50 and 5.00), NaOH concentration (0.1, 0.5 and 1.0 M), and time of each crosslinking treatment (3, 10 and 30 minutes) were studied. Each parameter was independently changed while the others are fixed, in order to understand their influence on mechanical properties of the dressings. The produced formulations are described in **Table 4.1**.

Table 4.1: Formulations to test in mechanical properties.

Type	[Polymer] (%)	[Ca] (%)	Time in $CaCl_2$ (min)	[NaOH] (M)	Time in NaOH (min)
A	2	2.50	10	0.5	10
B	2	5.00	10	0.5	10
C	2	2.50	30	0.5	10
D	2	2.50	10	0.1	10
E	2	2.50	10	1.0	10
F	2	2.50	10	0.5	30
G	3	2.50	10	0.5	10
H	2	1.25	10	0.5	10
I	2	2.50	3	0.5	10
J	2	2.50	10	0.5	3
K	3	2.50	10	0.5	30

Comparing each individual parameter in order to understand how it influences the Young's modulus, fixing the other variables, the following formulations, described in **Table 4.1**, were prepared in order to compare:

- **A and G:** Polymer concentration of 2 and 3%, respectively;
- **H, A and B:** $CaCl_2$ treatment concentration of 1.25, 2.50 and 5.00% respectively;
- **I, A and C:** Time in $CaCl_2$ of 3, 10 and 30 min, respectively;
- **D, A and E:** NaOH concentration of 0.1, 0.5 and 1.0 M. respectively;
- **J, A and F:** Time in NaOH of 3, 10 and 30 min, respectively.

Samples were produced in uniform cylindrical shapes of diameter 8 mm and thickness of 2 mm. These dimensions have been considered adequate, according to previous studies [89, 175, 176, 154].

Mechanical properties were determined by compression tests, recording the resistance of the samples to the compression of an aluminum cylinder probe with a diameter of 25 mm (for adequate compression tests, a probe with 2 to 3 times the diameter of the sample is required) [154, 175].

To perform the compression test, each sample was placed onto a plate and a compressive force was applied [154]. When the test was initiated, the probe descends until it touches the sample. At this time, the device starts to record the data. The probe then exerts pressure down to the defined value and, when the test is finished, it returns to its initial position.

The used settings in the software were decided according to some previous works [154, 89, 175], and are the following:

- **Test mode:** Compression;
- **Pre-test speed:** 60.00 mm/min (this is the speed of the probe until it reaches the sample);
- **Test speed:** 1.00 mm/min (it is the compression rate; this value is the usual for samples with this thickness [154, 89, 175]);
- **Post-test speed:** 600.0 mm/min (speed after the test is finished, at which the probe returns to the initial position);
- **Target mode:** "Distance" (the user can choose the distance that the probe travels since it touches the sample);
- **Distance:** The distance was chosen in order to the samples be compressed up to 80% of their original thickness;
- **Trigger type:** Auto (Force) (the trigger force is the force when the device starts collecting the data; it has to be sufficiently low to start when it touches the sample);

- **Trigger force:** 0.049 N (this value is pre-defined by the software as a value that is sufficient to the probe to sense the sample and start to collect the data);

Before performing a test, a calibration of force and height has to be carried out. The desired probe and accessories to perform the compression tests have to be placed on the right positions. Then, the parameters are chosen in the software. The sample is placed in position and the compression test is initiated. The software collects the data and a real-time "force *vs.* time" graph is displayed. When all the testes are finished, the data of force (N), time (s) and distance (mm) are collected.

The Young's modulus is calculated as the slope of the stress (MPa) *vs.* strain (%) curve within the linear region, which is the elastic region and is usually in a range between 0 and 15% of strain. For the current samples, the slope was calculated between 5 and 15% (more linear region and avoiding some instability in the beginning) [154, 89, 175].

In order to calculate the Young´s modulus, stress and strain have to be calculated before [161, 175].

Stress is calculated using equation 4.1:

$$Stress\ (MPa) = \frac{F(t)}{A} \tag{4.1}$$

where F(t), in N, is the applied force on the sample, and A, in mm^2, is the surface area of sample at the beginning of the experiment. Stress at each time, in MPa, or N/mm^2, is calculated by the force in each instant that is given by the *software*, and the surface area that was measured before the test. Mechanical stress is a measure of internal resistance exhibited by or material when an external force is applied. Strain is calculated using equation 4.2:

$$Strain\ (\%) = \frac{L_0}{L} \tag{4.2}$$

where L_0 is the initial thickness of the sample, that was measured before the test, and L is the quantity by which the length of the object changes. Mechanical strain is a geometric measure of deformation representing the relative displacement between particles in a material. The Youngs' modulus (E) is calculated using equation 4.3:

$$E\ (MPa) = \frac{stress}{strain} = \frac{F \times L_0}{A \times L} \tag{4.3}$$

As a consequence, it can be calculated by the slope of the stress-strain curve. Young's modulus, is a measure of the ability of a material to withstand changes in length when under lengthwise tension or compression. The stress-strain curve is created, calculating the stress and strain from the test data and from the dimensions measurements, and the slope is calculated between 5 and 15% of strain, as already described, calculating the Young's modulus for each sample. Finally, for each formulation present in **Table 4.1**, the average value is calculated from the 5 samples, with the respective statistical analysis.

After the mechanical tests, and according to the results (**Subsection 5.3.1**), in order to compare the influence of mechanical properties on other properties, as well as to further study the parameters that were significantly different in mechanical tests, the formulations J, F and K were characterized in the following tests: swelling, water vapor transmission rate, evaporative water loss and *in vitro* degradation. These tests are commonly used to test wound dressings. The same variables will be compared in all tests. As a result, the procedure to the sample preparation until freeze-drying, will be the same from here on.

Comparing each individual parameter in order to understand how it influences the properties that will be further evaluated, and fixing the other variables, formulations J, F and K were prepared to compare the effect of different values in the respective variables:

- **J and F:** Compare the influence of time of treatment with NaOH (3 and 30 min, respectively);
- **F and K:** Compare the influence of polymer concentration (2 and 3%, respectively).

In order to prepare the samples for the next characterization tests, pectin and chitosan were weighted and dissolved in the respective solution at the desired concentrations. The dissolved polymers were mixed at a 1:1 ratio and homogenized. The stock of the mixture was distributed by molds, in order to normalize thickness and surface area. The samples were treated with the adequate concentrations and times of $CaCl_2$ and NaOH, in the sequence previously decided, and then washed for 2 h with water, changing it each 30 min. Then, the samples were frozen at -20ºC overnight. Afterwards, they were freeze-dried for 24 h to be tested. Samples from the 3 formulations previously described were produced.

4.2.4.2 Swelling capacity

The ability to absorb exudates when in contact with skin wound surface is one of the major goals of wound dressings. Therefore, the determination of the swelling properties, such as water absorption (A_w) and equilibrium water content (EWC) of the wound dressings are critical. These parameters are important for quick absorption of exudates [151, 152, 154].

To perform these tests, cylindrical samples were produced with a diameter of 1 cm and thickness of 2 mm, according to the previously described procedure. 4 samples of each formulation were produced.

In order to perform the tests, the freeze-dried samples were weighted to determine their dry weight (W_d). Afterwards, each sample was immersed in 1 mL PBS (pH = 7.4) [151, 152, 154], and incubated at 37ºC for 24 h to reach equilibrium swelling [151, 154, 88]. At predefined time intervals, the samples were withdrawn from the PBS solution and the surface was blotted with paper to remove excess solution [154]. Then, samples were immediately weighed to determine wet weight (W_w) [88]. The defined time points were 30 min, 1, 3, 6, 12 and 24 h [152].

The absorption of water (A_w) was calculated using equation 4.4:

$$A_w\,(\%) = \frac{W_w - W_d}{W_d} \times 100 \tag{4.4}$$

where W_w is the weight of the sample in the wet state at a particular time t and W_d is the weight of the dry sample before swelling. The EWC was calculated using equation 4.5:

$$EWC\,(\%) = \frac{W_w - W_d}{W_s} \times 100 \tag{4.5}$$

An "Absorption of water (%) *vs.* time" graph was obtained for each formulation, by the average of 4 values.

4.2.4.3 Evaporative water loss

Wound dressings must maintain a moist environment at the wound site, which is considered ideal to promote wound healing, by controlling water loss at the wound site. The water loss from the pectin-chitosan dressings on exposure to the air was evaluated to examine its behavior when exposed to air. Therefore, the evaporative water loss (EWL) of each formulation was measured.

Cylindrical samples were produced with a diameter of 1 cm and thickness of 2 mm and 4 samples of each formulation were produced according to the previously described procedure.

Freeze-dried samples were incubated in PBS at 37ºC for 24 h in order to hydrate and reach the equilibrium swelling. After incubation, the samples were weighted and the initial weight (W_i) determined. Afterwards, samples were kept 37ºC. At predefined intervals, the samples were weighted. The defined time points were 30 min, 1, 3, 6, 12 and 24 h, in order to determine the weight at time t (W_t) [151], in order to calculate the loss of mass by evaporation.

The EWL of the samples was calculated using equation 4.6:

$$EWL\,(\%) = \frac{W_t}{W_i} \times 100 \tag{4.6}$$

where W_i and W_t are the initial weight after 24 h immersion and weight after time t, respectively.

A "EWL *vs.* time" graph was obtained for each formulation, as the average of 4 values.

4.2.4.4 Water vapor transmission rate

Wound dressings must hold an optimal water vapor transmission rate (WVTR) in order to maintain an adequate moist environment of wound site, which is ideal to promote wound healing, thus controlling water loss at the wound site. They must also be permeable to allow gaseous exchanges. For that purpose, WVTR tests were performed. The major problem in treating a burn is the loss of body liquid due to evaporation and exudation, which will decrease the body temperature and accelerate the metabolism rate. Therefore, the wound dressing must avoid or at least reduce the body liquid lost by controlling absorption and transmission, preventing dehydration, as well as by maintaining the humidity in the wound area to accelerate the formation of granulation tissue and

epithelialization. On the other hand, if the WVTR is low, accumulation of exudates will occur, which may cause the deceleration of healing process and opens up the risk of bacterial growth [151]. In the case of wounds, particularly burn wounds, the WVTR plays a key role with regard to moisture balance as it needs to be maintained throughout the repairing process. A low WVTR value could lead to numerous clinical challenges due to the buildup of exudates, whereas a very high value can lead to wound dehydration that decelerates the healing process [152].

Thin membranes of 1 mm of thickness and 1.5 cm of diameter were prepared. 5 samples of each formulation were produced according to the previously described procedure.

In order to perform these tests, Eppendorf tubes were filled with 500 μL of distilled water [151]. Then, each one was covered with a freeze-dried sample and gripped with parafilm for adequate isolation. This setup was weighted, in order to determine the initial weight (W_i), and then incubated at 37ºC for 24 h [151, 152]. At predefined time intervals, each setup was weighted again to determine the weight at time t (W_t). The defined time points were 30 min, 1, 3, 6, 12 and 24 h [151, 152].

In order to calculate the rate of water vapor transmission through the membranes, the WVTR was calculated using equation 4.7:

$$WVTR\,(g/m^2/h) = \frac{W_i - W_t}{t \times A} \tag{4.7}$$

where W_i is the initial weight, W_t is the final weight, t is time and A is the area of the Eppendorf tube mouth, by which the water valor passes through the membrane sample. W_i-W_t is the weight of water vapor transmitted through the membrane.

A plot of "WVTR *vs* time" was obtained, as the average of 5 values.

4.2.4.5 *In vitro* degradation tests

A wound dressing must be stable over time in order to continuously protect the wound. Therefore, the determination of the degradation of the samples *in vitro* is very important.

Cylindrical samples were produced with a diameter of 1 cm and thickness of 2 mm. 12 samples of each formulation were produced (4 samples for each formulation and for each time point), according to the previously described procedure.

The freeze-dried samples were weighed to record W_0. Then, they were immersed in 1 mL PBS (pH 7.4), and incubated at 37ºC for 14 days [155, 151, 156, 154, 89, 177, 178]. These conditions provide a similar environment to the one of wound exudate [178]. PBS is used to simulate the biological fluids, which is important to assess the degradation rate, but the degradation environment depends on the wound type. At different time points (3, 7 and 14 days), the biodegradability of the samples was tested by monitoring the weight changes. At each time point, the respective samples were removed from the solution, washed with ultrapure water 2 times for 30 min, frozen, freeze-dried for 24 h to obtain sponges again, and weighted (W_t) in order to calculate the degradation. All the samples were freeze-dried in the same freeze-drying cycle, in order to fix this variable. The PBS solution was renewed every 3 days by fresh solution. The mass change of the dried samples

serves to evaluate the degradability of the samples. An incubation time of 14 days is adequate to study the long-term behavior, stability and degradation of the samples [151, 178].

The degradation percentage is calculated by the weight loss, using equation 4.8:

$$Weight\ loss\ (\%) = \frac{W_0 - W_t}{W_0} \times 100 \tag{4.8}$$

where W_0 is the initial weight and W_t is the weight at time t, before and after degradation, respectively. The percentage of remaining mass of the samples due to degradation is calculated using equation 4.9:

$$Remaining\ weight\ (\%) = \frac{W_t}{W_0} \times 100 \tag{4.9}$$

A "Remaining weight (%) *vs.* time" graph was obtained for each formulation, as the average of 4 values.

4.2.4.6 Handling of wound dressings

From a clinical perspective, it is important that the health care provider can easily adjust the wound dressing to the characteristics of a wound. To this end, it is essential that wound dressings allow easy manipulation, can be cut into the desired shape and adapt to the wound site. A qualitative test was performed to evaluate the manipulation of the freeze-dried dressings as well as the ability to easily cut the dressings towards a specific shape. The adhesion to the moist skin was also qualitatively evaluated.

4.2.4.7 Statistical analysis

In order to perform the statistical analysis, the GraphPad Software was used. In order to compare whether there is a difference in the dependent variable for two independent groups, the Mann-Whitney was used. A difference is statistically significant when the p value is lower than 0.05. All the data were expressed as the mean ± standard deviation (SD).

Chapter 5

Results and Discussion

5.1 Pectin purification

After dissolution, the solution presented a pH value of 3.5 (thus the solution was acid), and then this value was successfully adjusted to 6. The pectin solution, after successive filtrations, presented adequate color and viscosity, usual for this type of solution. After freeze-drying, the solid pectin presented adequate color, density, porosity, and texture, and a sponge-like structure. No further tests were performed to verify the purification of pectin as the protocol is well-established and was previously validated in the group.

5.2 Qualitative optimization of wound dressing formation

5.2.1 Determination of the dissolution limit of each polymer

Observing the dissolution of each polymer in each concentration, and taking into account the homogeneity, ability to pipette and presence of solid particles, it was possible to conclude that the concentration limit in which pectin can be completely dissolved is 8%, while for chitosan this value is 6%. These polymers cannot be completely dissolved in values above these. Hence, concentrations above these values are not going to be used for further studies.

5.2.2 Determination of the best pectin-chitosan ratio

Comparing the 3 tested ratios, the 1:1 ratio was the one that presented better results, evaluating the mechanical properties and visual aspect of the gels, i.e., forming more consistent gels. The better results for this ratio may be because all the amino groups of chitosan interact with the carboxyl groups of pectin, by ionic interactions. This fact may lead to form a more stable and uniform gel.

Therefore, using a 1:1 ratio, and taking into account that the limit of dissolution of chitosan is 6% (as explored in **Section** 5.2.1), both polymers will not be dissolved at values above 6% from now on. When mixed, the solution between them have 3% of each polymer. This is the maximum

limit for the concentration of both polymers, due to the ratio used and the solubility limit of the polymers.

5.2.3 Determination of the polymer concentration to form a consistent and homogeneous hydrogel

With the objective of understanding the influence of the polymers concentrations on the gel formation, the following results were observed:

- 1% of both polymers in the mixture did not form a gel, and the mixture was a liquid solution, non-handleable. When the crosslinking agents were in contact with this solution, they mixed, and a freeze-dried sponge was not successfully produced. The not formation of a gel with this concentration may be because there are less available groups in the polymers to the crosslinking agents;
- 2% formed a gel with some consistency; thus, it is the minimum limit to obtain a gel with adequate properties;
- 3% also forms a gel, very similar to the 2% one, to the nacked eye;
- 4% was not tested (as it was already described in **Section** 5.2.2), because chitosan could not be completely dissolved at 8%, as required by the ratio 1:1.

Therefore, 1% was discarded, and the concentrations to use from now on are 2% and 3% of both polymers on the pectin-chitosan PEC. These mapped concentrations are the ones that are able to form a hydrogel with adequate mechanical properties. Due to the qualitative similarity between both concentrations, quantitative tests may be performed in order to compare them.

5.2.4 Determination of the optimal crosslinking methods for hydrogel formation

Upon mixing, the pectin-chitosan PEC is a viscous fluid, not forming a gel, because none of the polymers is crosslinked, and the only bonds between polymers are electrostatic interactions, which are weak bonds.

- **Pectin crosslinked in $CaCl_2$ followed by NaOH treatment**: When $CaCl_2$ contacted pectin, pectin changed from a fluid to a gel with some consistency. Then, after contact with NaOH, no changes were detected. This gel maintained the structural integrity upon incubation in distilled water. Hence, $CaCl_2$ is capable of crosslinking pectin, as expected;
- **Pectin in the presence of NaOH, followed by crosslinking in $CaCl_2$**: In NaOH, a fragile structure was observed, and some pieces started to dissociate from the main structure, which indicates that pectin has dissolved. When $CaCl_2$ was added, the structure continued disaggregating. This shows that a strong base or a high pH contributes to the depolymerization of pectin;

- **Chitosan in the presence of $CaCl_2$, followed by NaOH incubation**: When $CaCl_2$ contacted chitosan, the polymer maintained its fluidity. The explanation for the PEC gels only treated with $CaCl_2$ were not very consistent, may be because chitosan remained fluid after treatment with $CaCl_2$, so there is a need to also crosslink chitosan. However, when chitosan reacts with pectin, the consistency increases compared to chitosan alone, because electrostatic interactions form between them. Afterwards, when in contact with NaOH, little modifications were observed, but some consistency was gained (similar to chitosan only treated with NaOH, so it may indicate that $CaCl_2$ has no influence in chitosan). In water, no modifications were observed;

- **Chitosan treatment in NaOH, followed by $CaCl_2$**: Consistent gel was formed when chitosan was in contact with NaOH (NaOH crosslinks chitosan) and no alterations were observed after contact with $CaCl_2$. In water, no alterations were observed;

- **Pectin and chitosan in distilled water**: The pectin-chitosan PEC without any crosslinking treatment dissolved in water.

In summary, $CaCl_2$ is essential for the crosslinking. Otherwise, a consistent and easy to handle gel is not formed. Also, the PEC (only by blending pectin and chitosan) dissolved in water, which demonstrates that the electrostatic interactions (weak bonds) established in the pectin-chitosan PEC, only by blending the polymers, are not sufficient enough to maintain its structural integrity. The mechanical properties are low (as already described, the PEC without crosslinking formed only a viscous fluid and not a gel) and the mixture dissolved in water. Hence, it is needed to crosslink at least one of the polymer chains, forming a semi-IPN, or both of them, forming a full-IPN.

Using only $CaCl_2$ (which only crosslinks pectin), a consistent gel is formed, but with NaOH the mechanical properties are superior (the gel is harder and more consistent). In addition, NaOH also neutralizes the pH, which is important for contacting with cells and, therefore, for the biological performance of the dressings. Also, only with $CaCl_2$, the gel dissolves quickly in PBS (it was expected because there is an exchange of calcium ions with monovalent ions in PBS), showing that the electrostatic forces are not sufficient to sustain the gel structure. DMEM has many ions, such as calcium ions, and so gels only treated with $CaCl_2$ dissolve in PBS but not in DMEM. When a sample is immersed in DMEM, after being treated with NaOH, the medium turned pink-red, which did not occur when NaOH was not used. This showed that NaOH neutralized the pH, which was acidic to dissolve chitosan, which is important to cells. This color in this medium showed a physiological environment (as it was described in **Section 4.1.2**). Thus, treatment with NaOH is important for the final application, when applying the dressings in direct contact with cells, besides its use on the crosslinking of chitosan and in the improvement of the mechanical properties of the gels.

NaOH cannot be used alone as the PEC starts dissolution (because pectin dissolves in NaOH if not crosslinked before with $CaCl_2$, as described above).

Therefore, in this case, the crosslinking of both polymers is needed. $CaCl_2$ is a crosslinker of pectin and NaOH works as a crosslinker of chitosan, which is according to what is explained in **Section 2.2.3.5**. $CaCl_2$ is able to crosslink pectin carboxyl groups that were not bound to chitosan amino groups. NaOH crosslinks chitosan by reducing the repulsion between chitosan chains (basification step) that were created during its dissolution in an acidic environment.

In conclusion, the two methods are needed to form a full-IPN hydrogel (by the crosslinking of both polymers), and there is no need of additional chemical crosslinking agents, such as genipin or carbodiimide, since the two tested agents worked well, according to the goals. Furthermore, as already described in **Section 2.2.3.5**, chemical crosslinkers should be avoided.

Evaluating the sequences in the pectin-chitosan PEC instead in the polymers alone, the following results were observed:

- **$CaCl_2$ followed by NaOH**: Very consistent gel;
- **NaOH followed by $CaCl_2$**: The gel starts dissolving, which is consistent with the fact that pectin dissolves in NaOH before any other crosslinking treatment;
- **$CaCl_2$ and NaOH simultaneously**: Gel with little consistency. It may be explained by an immediate reaction of NaOH with pectin, which do not let pectin to crosslink with calcium ions.

According to these results, pectin should be treated with $CaCl_2$ before being treated with NaOH given that, in the opposing sequence, it starts to dissolve. Regarding chitosan, the order is seems to be irrelevant. Concluding, from now on, the used crosslinking methods will be $CaCl_2$ and NaOH, in this order.

Examples of obtained gels using this sequence are present in **Figure 5.1**.

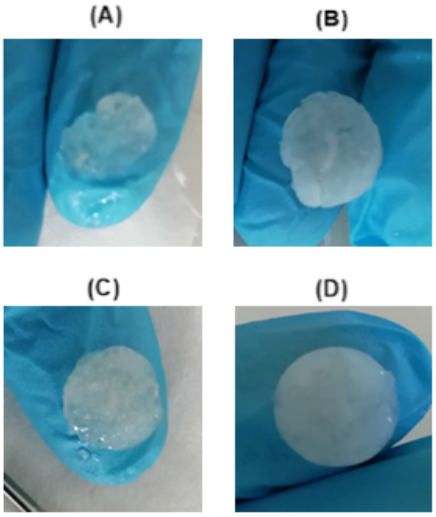

Figure 5.1: Gels. From (A) to (D) an increase of consistency and homogeneity of the gels can be observed. These different gels were obtained by variations in the parameters studied.

5.2.5 Determination of the concentration of each crosslinking agent

All the possible combinations formed a consistent gel, with little difference between them to be distinguished to the naked eye. Lower crosslinker concentrations led to softer gels. However, both $CaCl_2$ at 5.00% and NaOH at 1.0 M did not result in significant differences to the lower concentrations, since there may be a saturation of functional groups. As this test was inconclusive in a qualitative way, all the possible combinations were tested via quantitative characterization using mechanical testing. Since, no lower limit was found, any of these concentrations may be sufficient to produce a gel that, after freeze-drying, is capable of exhibiting adequate mechanical properties, so it has to be tested.

5.2.6 Assessment of the efficacy of crosslinking by stirring or diffusion

As the pectin-chitosan PEC is a very viscous fluid, it is very difficult to stir it. This difficulty increases with the addition of the crosslinking agents, as the crosslinking reactions are very fast. Hence, the diffusion method is the preferred one, as it presented good results, covering the PEC with the crosslinker and then letting it actuate. It was observed that when there was no adequate homogenization between the crosslinker and the polymer, the formed gel was easily disaggregated, so this homogenization has to be guaranteed.

5.2.7 Determination of the crosslinking time

Observing the results in a qualitative way, from 3 to 30 min, some differences could be noted regarding the consistency and manipulation, but from 30 to 45 min no difference was apparent. Therefore, from now on the times for each crosslinking treatment will be 3, 10 and 30 min.

5.2.8 Determination of the best step in the fabrication sequence to perform freeze-drying

All the samples were successfully freeze-dried, forming a consistent and homogeneous sponge that preserve the original shape. In addition, dressings were resistant to uniaxial and lateral compression with fingers, being easy to handle.

The observations of the 5 formulations (whose legend is in **Section** 4.2.3.8) are described below:

- **(1)** The already freeze-dried samples were treated with $CaCl_2$, and in this step, they presented a high volume increase, completely changing its disc shape, which is not desired. After 48 h incubation in PBS and culture medium, samples started dissolving;

- **(2)** After all the treatments ($CaCl_2$, freeze-drying and NaOH), the samples maintained their structure, consistency and shape. This formulation maintained the shape without dissolving;

- **(3)** After freeze-drying, these sponges (that were treated with $CaCl_2$ and NaOH before freeze-drying) are yellow. After 48 h incubated, both in PBS and culture medium, the samples start dissolving;

- **(4)** After freeze-drying, the sponges (that were also treated with $CaCl_2$ and NaOH before freeze-drying) were also yellow. After washing, the color changes to white. After 48 h incubated, both in PBS and culture medium, the samples start dissolving. Both (3) and (4) maintain the shape but are not very stable, which may indicate that NaOH remnants during freeze-drying may affect the process, and an additional washing step before freeze-drying may be needed. NaOH is probably the reason why these sponges are yellow after freeze-drying. The samples were treated with NaOH before freeze-drying, but not washed before that process. This may also seem that a washing step after freeze-drying does not exert any effect, because (3) and (4) only differ in this parameter and present the same behavior;

- **(5)** After incubation, the samples maintained their structure, consistency and shape. This formulation maintained the shape and did not degrade. Furthermore, these samples were treated with NaOH before freeze-drying, but are white. It may be concluded that a washing step to remove NaOH excess before freeze-drying is crucial to a well-succeeded freeze-drying process, resulting in sponges that do not degrade in culture medium nor in PBS. The washing step with deionized water is important to remove any nonreactive materials that were not incorporated into the network [142, 81, 167].

All the formulations, when treated with any solution, turn their sponge-like consistency in a more gel-like consistency. Both (2) and (5) maintained the shape, integrity and were stable after a 48 h incubation, but the ideal situation seem to be perform both crosslinking treatments at the same time instead of one before and the other after freeze-drying (what happens in (2)). Furthermore, if both crosslinking processes are performed before freeze-drying, it is easier to handle the dried sponges than treat the sponges with solutions and handle them wet and in a gel-like consistency. Therefore, formulation (5) is the preferred one.

Concluding, freeze-drying after treatments with $CaCl_2$ and NaOH with a washing step before freeze-drying, is the preferred method, presenting the best mechanical properties and shape maintenance. Therefore, this sequence was selected for further studies: $CaCl_2$ $\rightarrow$ NaOH $\rightarrow$ washing step of the gels (2 h, changing water each 30 min) $\rightarrow$ freeze-drying at -50ºC (24 h). The time of treatments with $CaCl_2$ and NaOH depends (it will be varied in further experiments to evaluate its impact on wound dressing properties), as explained in **Section 5.2.7**.

An example of a freeze-dried sponge is present in **Figure 5.2**.

5.2.9 Qualitative comparison of the degradation behavior of the samples before and after freeze-drying

When comparing the behavior of hydrogels and freeze-dried sponges in the performed tests, it was possible to verify that the tested formulations presented very similar consistency and degradation

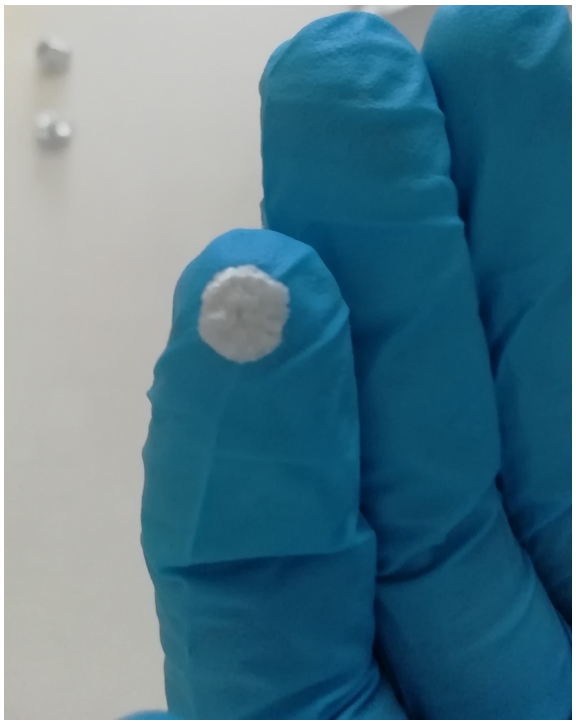

Figure 5.2: Freeze-dried sponge.

behavior when immersed in solutions of water, PBS and DMEM. Both gel and sponge without crosslinking treatments degraded in all 3 solutions after 24 h of incubation. Both gel and sponge only treated with $CaCl_2$ maintained their stability in water and DMEM, but started degrading in PBS due to ionic exchange with monovalent ions. Finally, both gel and sponge treated with both $CaCl_2$ and NaOH maintained their shape and structure after 24 h of incubation in all solutions due to the formation of a more crosslinked polymer network. Therefore, these data indicate the importance of forming a crosslinked gel, and consequently consistent and stable, that does not dissolve, in order to obtain an adequate and stable sponge structure after freeze-drying.

5.2.10 Qualitative optimization: main conclusions

After this qualitative optimization process, some conclusions may be drawn regarding the variables to fix and the ones to test and compare in quantitative tests. According to the studies carried out, and based on the reports already available and referred, the optimal conditions and parameters are:

- Pectin-chitosan ratio: 1:1 (established);
- Concentration of each polymer on the PEC: 2% or 3% (to further evaluate);
- Crosslinking sequence: $CaCl_2$ followed by NaOH (established);
- Crosslinking by diffusion (established);
- Treatment time (for each treatment): 3, 10 and 30 min (to further evaluate);

- $CaCl_2$ concentration: 1.25, 2.50 and 5.00% (to further evaluate);
- NaOH concentration: 0.1, 0.5 and 1.0 M (to further evaluate);
- Freeze-drying (-50ºC, 24 h) after both crosslinking treatments (with washing before freeze-drying) (established).

In **Figure 5.3**, the steps carried out in order to produce a sponge, from here on, are described.

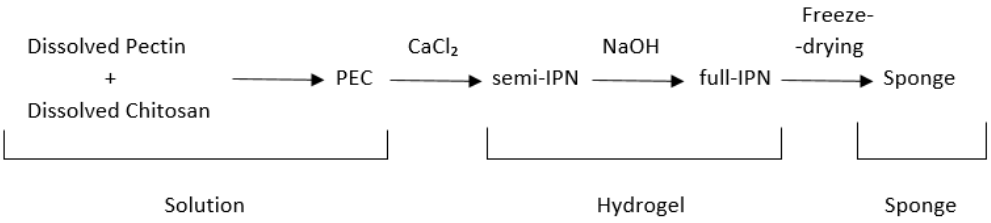

Figure 5.3: Sequence of steps to produce a sponge

All the possible combinations between these variables were tested, changing only one variable while the others were kept constant, in order to compare all the variables.

After having these parameters well defined and optimized, a protocol was created in order to test the variables in quantitative tests to assess the best formulation to produce a final product (wound dressing), comparing the different formulations between them and with the results in the literature, and to understand the influence of some properties in others.

Firstly, mechanical tests were performed using selected formulations, and then the most promising ones proceed to additional studies, or the ones that are interesting to evaluate the influence of mechanical properties in other properties.

5.3 Quantitative characterization of wound dressings

5.3.1 Mechanical tests

According to what was previously explained for mechanical tests (**Section 4.2.4.1**), 5 samples of each formulation (**Table 4.1**) were produced, in order to compare and understand the influence of each studied variable on the mechanical properties (2 and 3% of polymer concentration; 1.25, 2.50 and 5.00% of $CaCl_2$ concentration; 3, 10 and 30 min of time in $CaCl_2$, 0.1, 0.5 and 1.0 M of NaOH concentration; 3, 10 and 30 min of time in NaOH). All the tests were carried out in the same conditions (same day, temperature and freeze-drying cycle), so that variables can be compared without the influence of other conditions. A typical compression test is shown in **Figure 5.4**.

In order to calculate the Young's modulus, the stress-strain curve was obtained from the collected data, and then the slope in the region between 5 and 15% of strain was calculated. Afterwards, the Young's modulus of each formulation was calculated as the average value of the 5 samples.

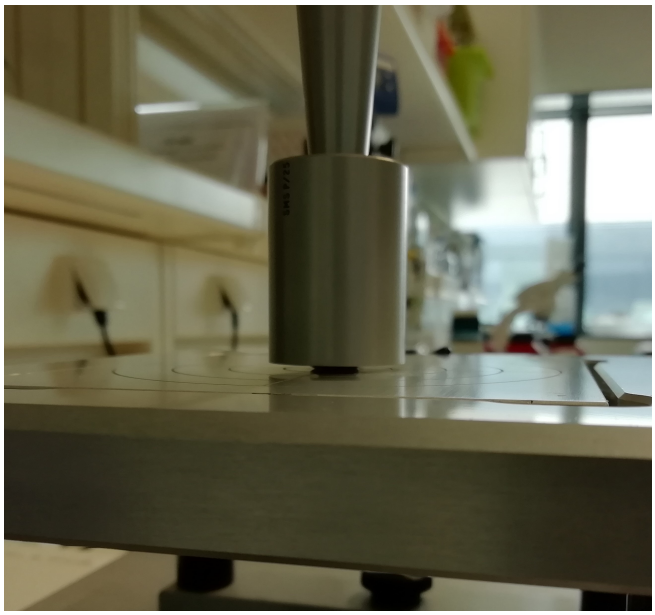

Figure 5.4: Compression test using the texture analyzer.

In **Table 5.1**, the tested formulations are described, as well as the variables to compare in each group of formulations and its values. Also, the average values of Young's modulus, standard deviation (SD) and R^2 obtained are described. The formulation A is repeated in the table because it was used to compare all the variables, as can be observed in the table. In **Figure 5.5**, the average Young's modulus for each formulation is shown, grouped by the variable to compare. The standard deviations are also indicated.

Table 5.1: Average Young's modulus (E), standard deviation (SD) and average R^2 of each formulation.

Formulation	Variable	Value	E (kPa)	SD	R2
A		2%	3.12	0.52	0.930
G	[Polymer]	3%	3.92	0.90	0.996
H		1.25%	2.66	0.69	0.932
A		2.50%	3.12	0.52	0.930
B	[$CaCl_2$]	5.00%	3.36	0.97	0.905
I		3 min	2.70	0.72	0.878
A		10 min	3.12	0.52	0.930
C	Time (in $CaCl_2$)	30 min	3.22	0.78	0.894
D		0.1 M	2.80	0.46	0.915
A		0.5 M	3.12	0.52	0.930
E	[NaOH]	1.0 M	3.38	0.65	0.829
J		3 min	2.60	0.64	0.954
A		10 min	3.12	0.52	0.930
F	Time (in NaOH)	30 min	5.68	1.58	0.811

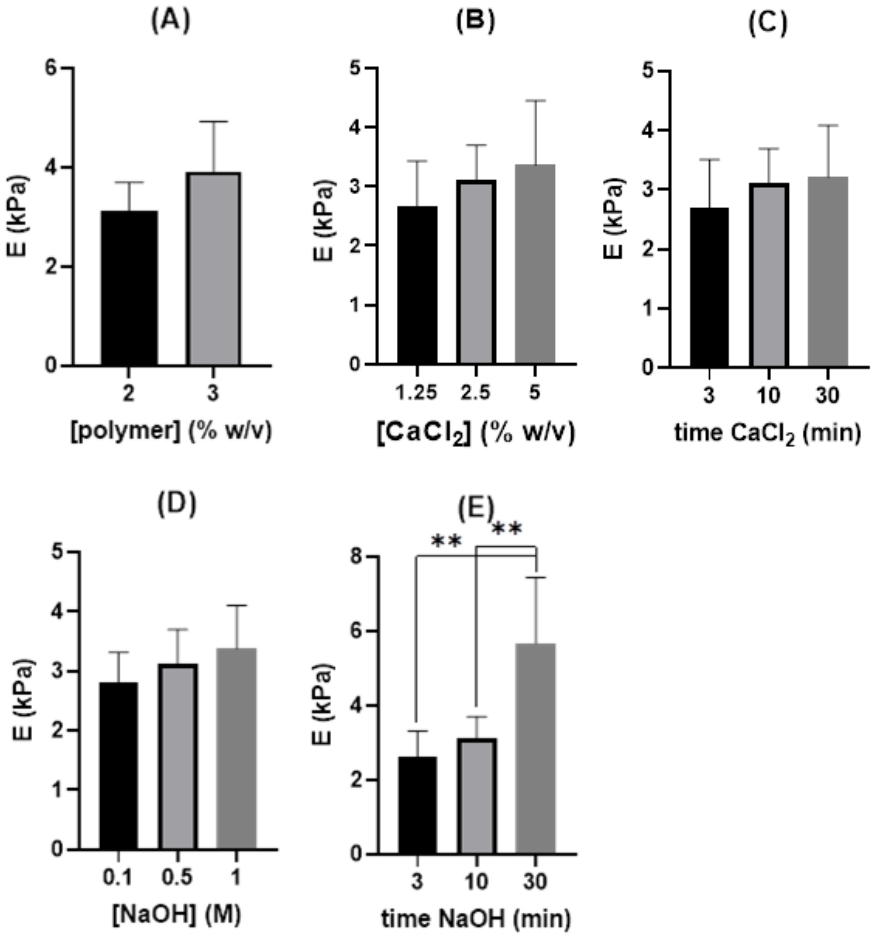

Figure 5.5: Effects on Young's modulus of all formulations of: (A) polymer concentration; (B) $CaCl_2$ concentration; (C) time in $CaCl_2$ treatment; (D) NaOH concentration; (E) time in NaOH treatment. The points represent mean ± SD (n = 5). ** - 0.01

According to what can be seen in **Table 5.1** and in **Figure 5.5**, and in the range of values tested for each variable, it can be observed that Young's modulus increases with higher:

- Polymer concentration;
- $CaCl_2$ concentration;
- Crosslinking time in $CaCl_2$;
- NaOH concentration;
- Crosslinking time in NaOH.

Analyzing the graphs, and supported by **Table 5.1** with the exact values, it can be observed that the time of treatment with NaOH (**Figure 5.3.1** (E)) is the variable that influences more markedly the mechanical properties (increase of 20% of the Young's modulus from 3 to 10 min and specially an increase of 82% from 10 to 30 min). The only significant differences were between the

conditions 3 and 30 min and 10 and 30 min, in **Figure 5.3.1** (E), as represented by the asterisks, presenting a p value lower than 0.05. Analyzing **Figure 5.3.1** (A), it can also be observed that the concentration of polymer represents some difference (26% of increase), but not statistically significant. On **Figure 5.3.1** (B), it can be observed that the modulus increases with the $CaCl_2$ concentration, but this difference seems to decrease this tendency from 2.50 to 5.00%, where the increase was only of 8% of the modulus, while from 1.25 to 2.50% was 17%. This may be caused by a saturation of functional groups to the crosslinker. On **Figure 5.3.1** (C), a similar situation may be observed, but in a more significant way: while from 3 to 10 min, the increase of the modulus value was 16%, the increase between 10 and 30 minutes was only 3%. This suggests that the crosslinking reaction with $CaCl_2$ is fast because from 10 to 30 min the difference is almost none. Analyzing **Figure 5.3.1** (D), it seems that, at least for the range of NaOH concentration tested, the increase of the modulus is almost the same among bars. However, as it can be seen, from 0.1 to 0.5 M the concentration increases 5 times and the modulus increases 11%, while from 0.5 to 1.0 M the concentration increases only 2 times and the modulus increases 8%, indicating that there is no direct proportionality. Thus, higher concentrations are expected to produce similar results. Also, concentrations of 1.0 M or above of NaOH may start affecting the cell viability [179]. In this regard, a published study, by Noriega *et al.* (2011) [179], has evaluated the impact of chitosan neutralization using different NaOH concentrations (0.1-1.5 M) on the cellular response (using chondrocytes). Results showed that NaOH concentrations of 1.0 M or above decreased the cell counting (reduced cell number).

As already discussed, only in the time of treatment with NaOH there is significant statistical differences (**Figure 5.3.1** (E)), namely between the 30 min treatment and the other times studied. The value of Young's modulus for this condition was the highest obtained, being 5.68 kPa, while the second higher was only 3.92 kPa (the only one with 3% of polymer concentration). On the opposite side, the lowest value was 2.60 kPa (3 min of treatment with NaOH). These results suggest that the main variable affecting the mechanical properties of the dressings is the time in NaOH, not only because is the only variable that presents significant statistical differences, but also because the highest (the stiffest dressing) and lowest (softer dressing) values of Young's modulus were obtained by variations only in this variable (3 and 30 min, respectively). Therefore, this variable will be studied in further tests, because of two reasons: the first one is to test the only variable that presented significant statistical differences, so more conclusive tests are needed; the other reason is to test the maximum and minimum value of the Young's modulus in the next tests (swelling, WVTR, degradation, porosity and evaporative water loss) in order to understand the how the mechanical properties influence in those properties.

Besides the two already described, one more condition will be studied. The chosen condition is the only condition with 3% of polymer concentration, due to 3 reasons. Firstly, it is interesting to study the influence of the polymer concentration, because it is a pivotal condition in every work, and some conditions may not strongly affect mechanical properties but may influence other properties (for example, a higher water absorption may not be due to a different value of the Young's modulus, but for example due to the content in NaOH). Secondly, according to the graphs

and the table, this is the variable with more differences between conditions among the ones that did not present significant statistical differences (modifications in the other variables seem to be more irrelevant, with similar values of Young's modulus). Lastly, the value obtained for this condition is the highest one (3.92 kPa) without counting with the one with NaOH at 30 min (5.68 kPa), which is much higher than the others (the lowest one among the other 8 values is 2.60 kPa).

In order to compare the incubation time in NaOH, the formulations with treatment of 3 and 30 min in NaOH (J and F), were further compared. In order to compare the polymer concentration, and using one of the two formulations previously described, another formulation was mechanically tested afterwards, with 3% of polymer and 30 min in NaOH (with 10 min in $CaCl_2$ at 2.50% and using NaOH at 0.5 M). This formulation (K) was not tested previously because there were no other formulations with only one variable to compare with it, but was the produced to compare polymer concentration. This formulation presented an Young's modulus of 6.63 kPa with a SD of 1.03 (as expected, the Young's modulus is the highest one because it holds a higher polymer concentration comparing to F, which was previously the one with a largest modulus). Therefore, in order to compare polymer concentration, F and K will be compared.

The human skin presents a Young's modulus between 4 and 10 kPa [180, 181] and the produced dressings are in this order of magnitude. As the dressings will be in direct contact with skin tissue, having a similar Young's modulus may be important for two reasons, namely (i) to not limit the patient's movements (and to avoid discomfort of a strange material) and (ii) so that the cells sense and interact with a material with similar stiffness. Therefore, the obtained values for Young's modulus can be adequate for a wound dressing.

If there was a need to improve mechanical properties, the tested variables could be increased: only polymer concentration (these polymers do not dissolve in higher concentrations) and NaOH concentration (concentrations of 1.0 M or above of NaOH may start affecting the cell viability [179]) should not be increased.

After the characterization of the mechanical properties, the following step was to understand how mechanical properties affect other properties, such as water absorption, degradation or cell viability. To this, the variables studied were polymer concentration (2 and 3%) and the time in NaOH (3 and 30 min), as already discussed. The other variables - $CaCl_2$ concentration, time in $CaCl_2$ and NaOH concentration - were fixed at 2.5%, 10 min and 0.5 M, respectively, because these values were already extensively studied in the mechanical tests.

The next tests were performed in order to answer some questions, namely: (i) Which formulation presents a higher value of the studied variable? (ii) The rate of the variable is constant throughout time? (iii) Do mechanical properties affect the studied variable? (iv) Do time in NaOH and polymer concentration influence the studied variable? (v) Do the studied formulations represent statistically significant differences for the studied variable? (vi) Are the values according to what is desired?

5.3.2 Swelling capacity

To determine the swelling kinetics, the mass of hydrogels was measured at 30 min, 1, 3, 6, 12 and 24 h of incubation. In **Figure 5.6**, the average weight of the 3 formulations along time is shown. In **Table 5.2**, the exact values for each time of each formulation are presented along with the SD.

Table 5.2: Average A_w and standard deviation (SD).

Formulation	Time (min)	A_w (%)	SD
J	30	703.41	58.91
	60	743.08	88.38
	180	846.95	89.88
	360	944.75	81.69
	720	1005.49	69.78
	1440	1037.31	97.67
F	30	851.09	84.64
	60	860.34	20.17
	180	986.01	65.92
	360	1022.45	15.94
	720	1099.44	62.13
	1440	1111.75	33.35
K	30	687.18	35.44
	60	743.86	26.78
	180	800.45	37.41
	360	851.14	35.87
	720	858.00	43.91
	1440	879.87	29.84

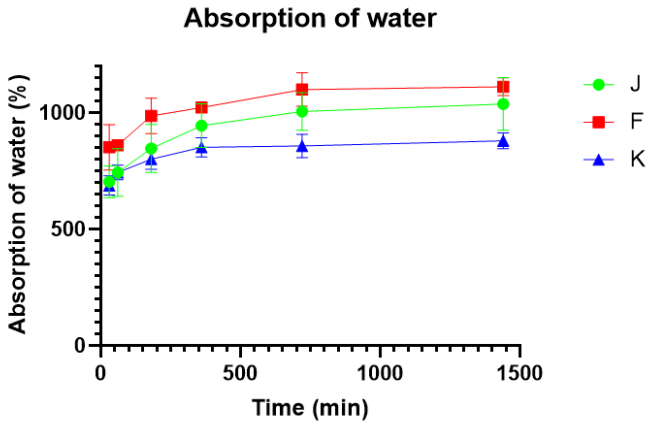

Figure 5.6: Water absorption of the tested formulations. The points represent mean ± SD (n = 4). J (2% polymer; 3 min NaOH); F (2% polymer; 30 min in NaOH); K (3% polymer; 30 min NaOH)

In the first 30 min, all the formulations presented the most absorption of water, followed by a slow increase in the absorption of water throughout the time. The equilibrium state was reached between 12 and 24 h. Thus, 24 h is confirmed as an adequate time for swelling tests. After that,

degradation (if any) may interfere with swelling. Therefore, the produced dressings are able to be used in a wound up to 24 h, absorbing water, being that this absorption is faster in the first 30 min.

The formulation that presented a higher capacity to absorb water is F (2% polymer; 30 min NaOH), while K (3% polymer; 30 min NaOH) presents the lowest capacity. In all time points, there is a statistically significant difference ($p < 0.05$) between formulations K (3% polymer; 30 min NaOH) and F (2% polymer; 30 min NaOH), making that the polymer concentration (2 and 3%) seems to influence the swelling capacity. Also, there is only a significant difference between F (2% polymer; 30 min NaOH) and J (2% polymer; 3 min NaOH) at 3 h. Therefore, the pairs J-K and J-F do not present significant differences between them. Therefore, the concentration of polymer presented significant differences in the swelling ability. On the other hand, the mechanical properties seem to not present a direct influence on the swelling properties, because J (2% polymer; 3 min NaOH) presents the lowest Young's modulus (2.60 kPa), while K (3% polymer; 30 min NaOH) presents the highest one (6.63 kPa), but they do not present a significant difference in the absorption of water. F (2% polymer; 30 min NaOH) presented an Young's modulus of 5.68 kPa.

As formulations J (2% polymer; 3 min NaOH) and F (2% polymer; 30 min NaOH) presented the highest swelling ability and they did not present significant difference for sweling, they are the most indicated to produce a wound dressing among the ones tested (treatment time of 3 and 30 min and NaOH, using always a 2% concentration of polymer) regarding absorption of water. Crosslinking is often measured by swelling tests: the more crosslinking, the less swelling is attainable [155]. So, according to these results, J (2% polymer; 3 min NaOH) is slightly more crosslinked than F, but the only difference between them is that J (2% polymer; 3 min NaOH) was treated with only 3 min in NaOH, while F (2% polymer; 30 min NaOH) was 30 min, so this must be further studied, by studying for example the effect of pH on pectin chains, or studying the chemical composition. Too much time in NaOH may somehow affect pectin chains, so an optimal time may exist. A compromise must be reached between swelling ability (lower crosslinking) and sufficient crosslinking to maintain a structure with enough integrity and resistant to degradation. Porosity may also influence swelling [155], with more compacted structures having lower water absorption, so SEM can be performed to confirm the results.

The absorption of water is very fast at the beginning, reducing the speed with time, as expected [151, 152, 154], being more useful in wounds where larger volumes of exudates have to be rapidly absorbed. After 24 h, formulation J (2% polymer; 3 min NaOH) presents 1037.31%, F (2% polymer; 30 min NaOH) presents 1111.75% and K (3% polymer; 30 min NaOH) presents 893% of water absorption. After 30 min, these values are 703,41, 851.09 and 687.18%, respectively. Therefore, these dressings may be more beneficial to wounds with more exudates in early-stage wound. However, it depends on the type of wound to which the dressings will be applied. Dressing materials need to show optimized level of fluid absorption ability in order to eliminate extreme exudates [89]. Therefore, the absorption property of wound dressing is an essential factor as it can promote the local hemostasis at the open wound and subsequently remove the wound exudates which can delay the wound healing and macerate surrounding skin [141].

The produced dressings showed the ability to absorb exudates and maintain a moist environment for wounds, especially great volumes at the first 30 min. The high swelling ratio and water absorption of the dressings indicated suitability for absorbing wound exudates and maintaining a moist wound healing environment.

In summary, all the tested formulations are able to absorb and retain a considerable amount of water after 24 h, providing a moist environment to the wound, which is essential to the wound healing process. Moreover, it is possible to conclude that between formulations J (2% polymer; 3 min NaOH) and F (2% polymer; 30 min NaOH) it is irrelevant the formulation that is used to produce dressings, since no significant differences were observed. Finally, it is possible to conclude that the mechanical properties have no significant influence swelling capacity, neither time in NaOH, while polymer concentration influences water absorption.

5.3.3 Evaporative water loss

After 24 h of incubation, 4 samples of each one of the 3 formulations were kept at 37ºC in order to measure the mass loss due to evaporation at 30 min, 1, 3, 6, 12 and 24 h. In **Figure 5.7**, the average of the remaining weight of the 3 formulations along time is shown, while in **Table 5.3**, the exact values for each time of each formulation are represented.

Table 5.3: Average remaining weight and standard deviation (SD).

Formulation	Time (min)	EWL (%)	SD
J	30	92.27	0.55
	60	86.16	0.48
	180	78.98	0.70
	360	72.58	1.84
	720	62.09	3.31
	1440	48.46	7.89
F	30	94.77	0.75
	60	89.00	0.55
	180	82.24	1.66
	360	75.32	1.88
	720	65.57	3.03
	1440	54.46	4.79
K	30	94.14	1.04
	60	88.97	1.61
	180	80.00	3.51
	360	72.60	4.97
	720	58.71	7.44
	1440	46.34	6.67

Analyzing the graph, it can be observed that the loss of water was approximately 5% after 30 min for the 3 formulations, and within 24 h it increased up to 50%. So, after one day, the samples retained about 50% of the absorbed water. Therefore, it is clear that the material holds a considerable amount of water when exposed to air under dry conditions during short periods,

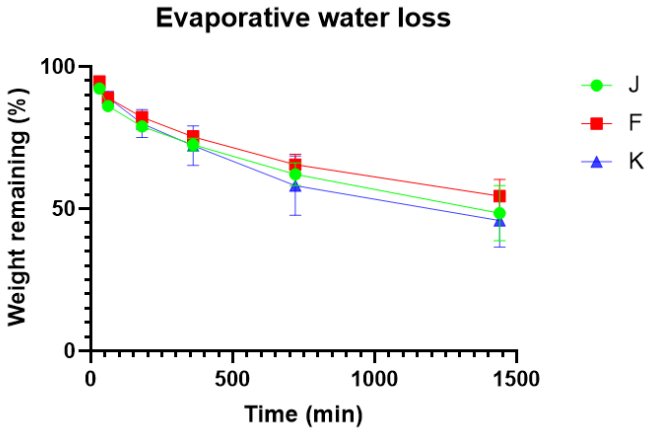

Figure 5.7: EWL of the tested formulations. The points represent mean ± SD (n = 4). J (2% polymer; 3 min NaOH); F: (2% polymer; 30 min in NaOH); K: (3% polymer; 30 min NaOH)

being that after 24 h the water content is still considerable. This is particularly important when the exudate is completely absorbed or the dressing is not externally hydrated, which can lead to the drying of the wound bed, and thus, a moist environment must be maintained in the wound bed to stimulate wound healing. Therefore, the dressings must retain a considerable amount of water to provide a moist environment to the wound, which is observed.

Dressings that, for example, retain only 5-10% of water after 6 h may be more beneficial to wounds with more exudates in early-stage wound, quickly removing the exudate, and then have to be replaced [151]. The dressings produced in this work seem to be adequate for various types of wounds (that are constantly producing exudate or that produce exudate only in early stages), since they are able to retain water for a long period of time, providing a moist environment to the wound and avoiding its drying. It has to be taken into account that the choice of the dressing strongly depends on the wound type.

The influence of hydrogel composition on the water loss was the same observed for swelling tests: F (2% polymer; 30 min NaOH) loses water in a slower way, while K (3% polymer; 30 min NaOH) loses faster (the one that absorbed water faster is the one that lost water slower, i.e., the one that presents higher capacity to absorb water is the one holding higher capacity to retain it). In all time points, even in 24 h, there are no statistically significant differences between all formulations ($p < 0.05$). As a consequence, Young's modulus, time in NaOH and polymer concentration do not affect this property. According to this test and swelling study, the formulation F (2% polymer; 30 min NaOH) is the one with higher capacity to absorb and retain water, even though not presenting a statistically significant difference when compared to other dressings - J (2% polymer; 3 min NaOH). Although the differences between formulations are statistically not significant, it is possible to observe a tendency for an increased difference between the dressings along time.

No equilibrium was apparently achieved after 24 h (the graph does not stabilize in a constant value for any formulation), which was expectable since the tendency is to continue loosing water.

Therefore, longer times do not need to be tested.

In summary, all the tested formulations were able to retain a considerable amount of water after 24 h, providing a moist environment to the wound and preventing water loss at the wound site, which is essential to the wound healing process, which was desired. Moreover, it is possible to conclude that, regarding the evaporative water loss, it is irrelevant the formulation that is used to produce dressings, since no significant differences were observed for this property between all the tested formulations. Finally, it is possible to conclude that the mechanical properties have no influence in this property, as well as time in NaOH and polymer concentration.

5.3.4 Water vapor transmission rate

To measure the WVTR, the water mass change was measured at 37ºC at different time periods (30 min, 1, 3, 6, 12 and 24 h) in order to determine the influence of dressing composition on this property. In **Figure 5.8**, the average of the weight of the 3 formulations along time is shown and **Table 5.4** presents the exact values for each time of each formulation.

Table 5.4: Average WVTR and standard deviation (SD).

Formulation	Time (min)	WVTR (g/m^2)	SD
J	30	6.71	1.43
	60	8.27	2.20
	180	20.02	7.62
	360	82.36	6.02
	720	107.59	11.78
	1440	198.88	15.32
F	30	7.25	0.66
	60	7.26	0.67
	180	30.17	5.41
	360	91.82	7.19
	720	125.83	10.19
	1440	221.60	18.09
K	30	6.65	2.02
	60	6.66	2.03
	180	26.71	10.06
	360	83.30	14.80
	720	115.79	22.90
	1440	214.38	32.48

In the first 6 h, the WVTR is faster, while from 6 to 24 h, the weight of water that passes through the membranes is slower (some membrane saturation may be present after 6 h). However, the WVTR behavior seems to present a linear correlation with time, showing that the produced dressings are permeable to water at least in the first 24 h after covering the wound. The curve was the expected, i.e., almost linear [152], and the rate is almost the same over time. After 24 h, the WVTR were (in g/m^2/day): 198.88 for J (2% polymer; 3 min NaOH), 221.60 for F (2% polymer; 30 min NaOH) and 214.38 for K (3% polymer; 30 min NaOH). The WVTR of normal skin is

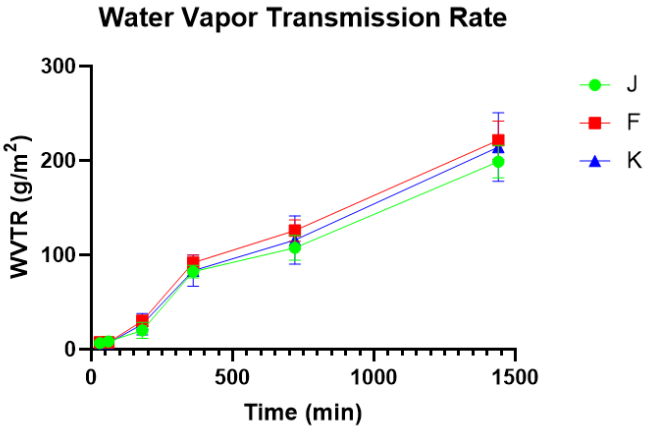

Figure 5.8: WVTR of the tested formulations. The points represent mean ± SD (n = 5). J: (2% polymer; 3 min NaOH); F: (2% polymer; 30 min in NaOH); K: (3% polymer; 30 min NaOH)

around 204 g/m^2/day [154, 173]. Therefore, the obtained values are very similar to the normal skin value, so it can be concluded that the produced dressings present adequate values of WVTR. Also, the WVTR of dressings from other works are between 200 and 500 [154, 173, 88], which is according to the obtained results. As already mentioned, WVTR value cannot be too high or too low.

Among the 3 tested formulations, F (2% polymer; 30 min NaOH) is the one that presented a faster WVTR, while J (2% polymer; 3 min NaOH) presented the slowest one. However, as already described, all of them present a very similar value to the one of normal skin. In all time points, there is no a statistically significant difference ($p < 0.05$) between any formulation. As a consequence, there is very little difference in the rate that the different formulations allow water passage. Therefore, the time in NaOH, polymer concentration and Young's modulus seem to not significantly affect WVTR.

The produced dressings showed the ability to be permeable to water, allowing gaseous exchanges, and at the same time enabling an adequate moisture environment for optimal healing, and the obtained values were similar to the WVTR of skin.

5.3.5 *In vitro* degradation

The *in vitro* degradation was monitored upon the incubation of dressing samples in PBS by measuring the weight changes at day 3, 7 and 14. In **Figure 5.9**, the average of the weight of the 3 formulations along time is shown. In **Table 5.5**, the exact values for each time of each formulation are represented.

After 14 days, dressing J (2% polymer; 3 min NaOH) maintained 93.60%, F (2% polymer; 30 min NaOH) maintained 91.51% and K (3% polymer; 30 min NaOH) maintained 95.21% of its original weight. The degradation rate was almost constant for all formulations, but it was faster from day 0 to 3 and more constant from day 7 to 14. Therefore, the degradation is faster in the beginning and it slows down over time. This degradation may be observed by the slope between

Table 5.5: Average degradation and standard deviation (SD).

Formulation	Time (days)	Remaining weight (%)	SD
J	3	96.90	2.53
	7	94.73	2.77
	14	93.60	2.79
F	3	96.14	3.83
	7	93.59	2.20
	14	91.51	2.76
K	3	98.01	0.93
	7	96.04	1.93
	14	95.21	0.95

points, but may be quantified according to the table: average weight decreased 2.98% between days 0 and 3, 2.34% between 3 and 7 and 1.40% between 7 and 14 for all formulations. The slope, that represents the loss of mass, is almost the same for all formulations in the different time points.

After 24 h, K (3% polymer; 30 min NaOH) degrades less while F (2% polymer; 30 min NaOH) degrades more, which is in accordance to the swelling and EWL results, where F (2% polymer; 30 min NaOH) was the one with more swelling and less loss of water by evaporation, in contrast to K. Therefore, in conclusion based in these 3 tests, K (3% polymer; 30 min NaOH) is the more crosslinked formulation (as expected, due to higher polymer concentration - 3%, and treatment time in NaOH - 30 min), while F (2% polymer; 30 min NaOH) is the less crosslinked (which must be further studied, as already discussed, because F (2% polymer; 30 min NaOH) has a treatment of 30 min in NaOH, while J (2% polymer; 3 min NaOH) is only treated 3 min). The one that absorbed and retained more water (F) is the one that degrades faster, while the one that absorbs and retains less water (K) is the one that degrades slower. Furthermore, in terms of degradation, K (3% polymer; 30 min NaOH) is the one that degrades less, making it the most adequate, since degradation is not desired and it is avoidable for this specific application (a wound dressing must be stable over time, in order to continue protecting the wound). Therefore, a compromise may be achieved regarding the desired application: more absorption and retaining of water or less degradation. However, in all time points, there are no statistically significant differences ($p < 0.05$) between any of the formulations tested. As a consequence, mechanical properties, time in NaOH and polymer concentration did not significantly affect degradation rate, being that all 3 formulations present a very similar degradation rate.

The produced dressings showed very little degradation in PBS, even after 14 days, so they can be used as long-term dressings, as it is desired, but may also be used as short-term. They can be used during different time periods, depending on the desired application. Also, the wound environment depends on the wound type (for example, acute or chronic), although PBS was always used because it simulates a typical body fluid. However, future studies must consider the use of wound simulated fluids to better mimic the real environment of the application. Furthermore, for degradation tests, human enzymes, such as lysozyme, could be used in PBS. The solution would have to be daily refreshed to ensure continuous enzyme activity.

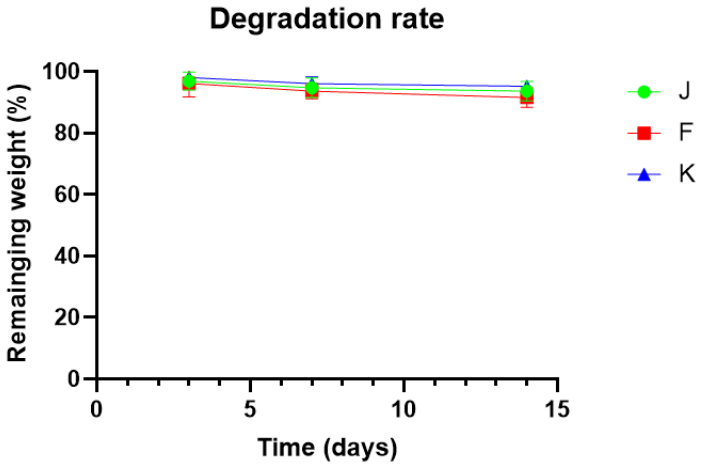

Figure 5.9: Degradation of the tested formulations. The points represent mean ± SD (n = 4). J: (2% polymer; 3 min NaOH); F: (2% polymer; 30 min in NaOH); K: (3% polymer; 30 min NaOH)

Concluding, all the 3 formulations presented adequate swelling properties, EWL, WVTR and degradation rate, being that only swelling presented statistically significant differences between formulations. F (2% polymer; 30 min NaOH) presented more absorption of water, less water loss by evaporation, more WVTR and faster degradation, although not significantly. On the other hand, K (3% polymer; 30 min NaOH) presented less swelling, more water loss by evaporation and less degradation, although also not significantly. Only WVTR seem to not present a direct proportionality with the other properties. Also, mechanical properties seem to not present a direct impact in these properties, because different Young's moduli did not lead to statistically significant different values of these properties. Furthermore, even though time in NaOH was the property that led to more differences in mechanical properties, polymer concentration was the property that led to more differences in the other studied properties.

All the formulations can be used, with adequate properties, due to the similarity between them, but their use depends on the application. F (2% polymer; 30 min NaOH) may be preferred when more absorption and retaining of water is needed, as well as more water vapor transmission rate, but K (3% polymer; 30 min NaOH) may be preferred when less long-term degradation is needed. While F (2% polymer; 30 min NaOH) can be more used in wounds that need the absorption of large volumes of exudates while preventing dehydration, K (3% polymer; 30 min NaOH) may be more used to only cover the wound for long times, protecting it from infection, without degrading. Depending on the need of using a softer or stiffer dressing, the different formulations may also be chosen: if a softer one is desired, formulation J (2% polymer; 3 min NaOH), with a Young's modulus of 2.60 kPa, may be preferred. If a stiffer one is desired, formulations F (2% polymer; 30 min NaOH) and K (3% polymer; 30 min NaOH), with Young's moduli of 5.68 and 6.63 kPa, respectively, may be preferred.

5.3.6 Handling of wound dressings

The produced dressings could easily be cut with a scissor to a desired shape, to be manipulated to apply to irregular shapes, presented ability to being adapted to moist skin, as well as to being hydrated. These properties are critical for the use of these dressings by a health care provider. The final product can be seen in **Figure 5.10**. The dressing exhibited good physical integrity and flexibility. Qualitatively, they adhered well to the moist skin, which is desired for a wound dressing. In the future, quantitative and standardized tests should be performed in order to evaluate the adhesion of these dressings to the skin. It was expected, since chitosan presented adhesive properties, as already addressed.

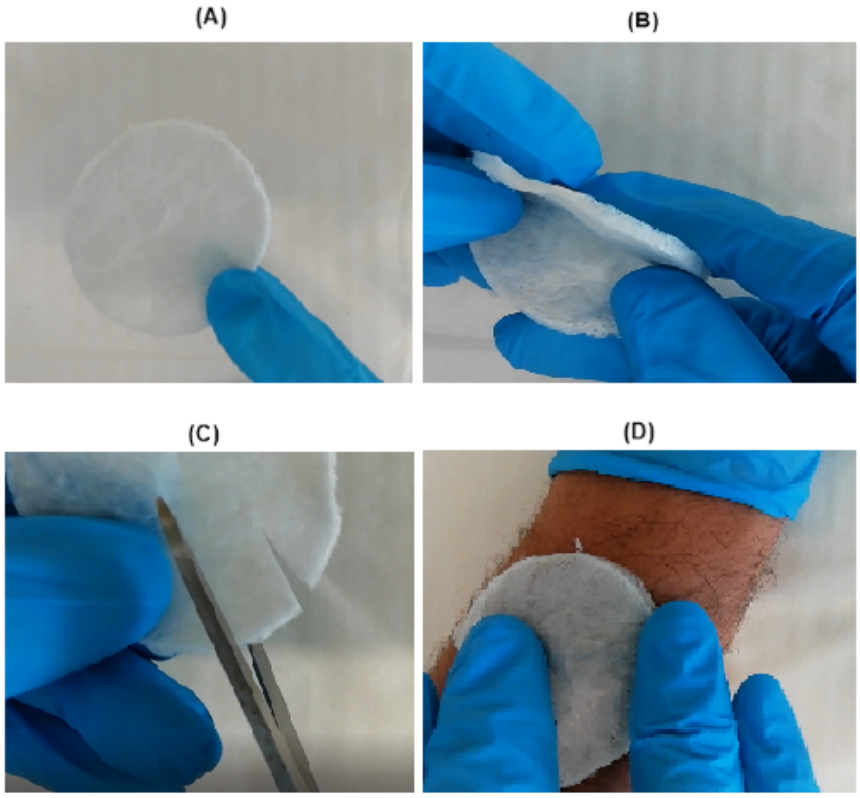

Figure 5.10: Wound dressing final product. (A) Round wound dressing; (B) ease of handling of the dressing; (C) Dressing cut; (D) Application on skin.

Chapter 6

Conclusions and future work

In this Chapter, the main conclusions of the research project are provided and perspectives for future work briefly discussed.

6.1 Conclusions

Wound dressings must fulfill some key requirements, such as biocompatibility, adhesion to the wound bed, flexibility, robustness, water absorption ability, allow gaseous exchanges, be easy to handle, among others. These properties were evaluated in detail, and the produced dressings presented adequate properties.

The dressings exhibited adequate and adjustable properties, and good physical integrity and flexibility. They showed to be robust and easy to handle, presenting also adequate mechanical properties, water absorption, degradation and permeability properties.

Mechanically, the dressings showed Young's modulus similar to the human skin, and were easy to handle and flexible, which is important to be cut and adapt to the wound contours. They also presented a good adhesion to moist skin as evaluated by qualitative preliminary test, being thus able to protect the wound. This Young's modulus is important to guarantee an identical environment to human skin and at the same time assuring the integrity of the structure is maintained.

Dressings were able to absorb and retain water, being also able to regulate the water loss, which is essential to maintain a moist wound environment to stimulate the healing process. Even after hydration, dressings presented the ability to retain a larger quantity of water after 24 h exposed to air, which is important to avoid dehydration. Also, they showed the ability to be permeable to water, allowing gaseous exchanges, thus enabling an adequate moisture environment for optimal healing, and the obtained values were similar to those of skin. The produced dressings showed the ability to be permeable to water, allowing gaseous exchanges and exhibiting WVTR values similar to the of skin.

Lastly, they maintained their structure after 14 days in PBS, with almost no degradation.

Concluding, the dressings presented the required physical properties for a wound dressing material.

All the 3 formulations that were studied in all the tests showed very similar properties, showing that variations in mechanical properties, time in NaOH and polymer concentration did not represent statistically significant differences in the evaluated properties. However, according to the desired application, the most adequate formulation can be selected. All the formulations can be used, with adequate properties, due to the similarity between them, but depending on the application, the formulations that presented more swelling capacity may be used when more absorption and retaining of water is needed, for instance in wounds that need the absorption of large volumes of exudates but preventing dehydration, as well as more water vapor transmission rate, but formulations that degrade less may be preferred when less long-term degradation is needed, only to cover the wound for long times, protecting it from infection, without degrading. Depending on the need of using a softer or stiffer dressing, the different formulations may also be chosen. A compromise must be established between all these properties.

6.2 Future work

In order to obtain more results on the produced dressings, some of the variables that were studied in the optimization process should be quantitatively characterized. More concentrations of $CaCl_2$, NaOH and polymers should be tested (intermediate values of the ones tested), allied to quantitative tests, in order to find the optimal conditions in a quantitative way.

In order to consolidate the data of the characterization tests performed, more independent assays should be performed for each test.

Another important step would be the increase of the number of samples tested in each characterization test, in order to consolidate the data, allowing the removing of outliers, reduce the SD and reduce discrepancy between results. More tests should be carried out in order to have more data, having a larger n, in order to consolidate the data and to improve statistical validity.

An interesting study would be the comparison of the produced samples with commercially available wound dressings (using them as a control group), with the same equipment and with the same methods, in order to compare them and understand the advantages that may differentiate.

Some additional material characterization, namely concerning physico-chemical properties, could be performed. In order to analyze the chemical structure of the samples and to confirm bindings between the polymers, and assess viability, Fourier transform infrared (FTIR) spectroscopy could be carried out. This technique identifies absorption bands on vibrational molecular transitions [141, 152, 154, 148]. X-ray diffraction (XRD) could also be performed in order to evaluate the structure of materials [156, 89]. Scanning electron microscopy (SEM) would be very useful to analyze surface morphology, topography and porosity of the samples. It would be interesting to compare the SEM results with those of swelling and crosslinking degree, to assess if the results are in agreement. Also, determination of surface roughness would also be interesting [152, 143].

The measurement of the porosity of the samples through liquid displacement method would be valuable as well, since the porosity of a wound dressing is crucial. The supply and exchange of oxygen through the pores of the dressing material are essential factors in wound healing. The

porosity of a material also enhances cell migration and the exchange and ability to absorb the excess exudates at the wound site. A material with high porosity is a potential wound dressing material [89], although this should not be the main requirement without all others. Compare the porosity results with SEM would be interesting.

Several biological tests could be also performed, in order to assess the influence of these properties on cell behavior. Firstly, in order to test cells in these dressings, a sterilization process must be carried out, immersing in ethanol at 70% for 1 h, followed by washing with ultrapure water or culture medium. Afterwards, *in vitro* cytotoxicity studies could be performed in order to evaluate cell viability, metabolic activity and the biocompatibility of dressings. In order to assess if the produced dressings are biocompatible and able to stimulate cell proliferation, assays involving the evaluation of dressings cytotoxicity and proliferation in the presence of relevant cells (e.g., dermal fibroblasts) should be performed. Furthermore, further biological tests could be performed to assess if they stimulate cell proliferation and tissue regeneration. Some further biological tests could be performed in order to study the influence of NaOH on cell viability, besides in the already addressed mechanical, swelling and degradation properties. Another example would be study the effect of mechanical properties on cell behavior and proliferation. Other parameters, such as topography, porosity and surface modifications are also known to influence cell behavior. Antibacterial effects of the prepared wound dressings could also be evaluated. Hemocompatibility of wound dressings could also be assessed by *in vitro* hemolysis assays (evaluating the blood coagulation activity). This would be important because dressings are in contact with blood. Some tests may also be performed to evaluate the adhesion strength of the dressing to the wound.

Ex vivo tests could also be performed, using skin excised from an animal. *In vivo* wound healing animal studies could be carried out, to evaluate the wound closure rate by histological observation, in order to assess the ability of the produced dressings to promote wound healing.

In terms of process, bioprinting could be used to produce a dressing with a well-defined structure, and in a more reproducible way.

In order to approve the produced dressings to biomedical applications, sterilization process must be studied.

These dressings were prepared in order to have adequate properties to be used as wound dressing, such as biocompatibility (the polymers used and the concentrations of the crosslinking methods are biocompatible), swelling, vapor transmission, degradation and mechanical properties. However, in the future, alternative procedures could be carried out in order to produce wound dressings that, besides protecting the wound, are able to stimulate wound healing, namely repair and regeneration. Also, production of dressings that could perform additional interesting tasks, such as drug delivery, by incorporating bioactive compounds, would be relevant. Besides working as a protecting material, it could additionally deliver bioactive substances. The addition of cells to the dressings could be interesting, because besides stimulating the formation of new tissue in a direct way, improving new tissue formation, they also stimulate the production of cell signaling factors and cytokines. Furthermore, it would be interesting to assess if the polymers require chemical modification in order to improve cell behavior. Finally, some methods such the release of drugs

may be used in order to stimulate cell proliferation (the maintenance of an adequate environment also stimulates proliferation, but this is not a differentiating factor from other dressings).

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