Macrophage activation by and degradation studies of the Skin2 dermal matrix

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Abstract

Autograft skin transplantation is still the standard treatment for severe skin loss, despite its limitations. Although competent regenerative therapies have been developed and commercialized, they still present anatomical, functional and aesthetic limitations. This dissertation addresses the development of the dermal layer of a biosynthetic skin substitute: Skin2.

The goal of this dissertation was to study the interactions between monocytes, which would later differentiate into macrophages, and cell scaffolds produced in-house via electrospinning, namely: THP-1 monocytic cell line viability and macrophage differentiation and polarization in inflammatory or regenerative (M1 or M2) phenotypes when seeded in the scaffolds, which were produced using different materials and mixtures of these materials; macrophage morphological differences amongst the different scaffolds; macrophage generated erosion on these scaffolds.

The materials used in electrospinning were gelatin from cold water fish skin (GEL), chitosan (CS), and Poly-ε-caprolactone (PCL), either singularly or mixed with each other in binary or ternary mixes. The scaffolds produced were then crosslinked with glutaraldehyde (GTA) if they had CS or GEL in order to maintain their physical properties, and seeded with THP-1 cells under two conditions: with and without the addition of phorbol-12-myristate-13-acetate (PMA), which induces monocytes differentiation into macrophages.

Viability results show that all materials are viable, and in GEL that underwent dehydrothermal treatment (DHT) and PCL, there is an increase in viability from 48 to 120 hours of culture, suggesting prolonged viability. Additionally, adhesion rates estimation shows that polymer blends tend to promote more cell adhesion compared to single polymer scaffolds.

Regarding macrophage generated erosion on the scaffolds, nothing could be observed under Scanning Electronic Microscopy (SEM). However, SEM results provided a clear observation of macrophages morphology and allowed to estimate their phenotype, clearer than fluorescence microscopy. Addition of PMA mainly induced a regenerative (M1) macrophage morphology, with cells presenting a round shape. When PMA is not added, the predominant phenotype morphology is M0 (inactivated) for unitary scaffolds and for CS GEL, and M1 for CS PCL, GEL PCL, and ternary mix.

Keywords: Polymers, Electrospinning, Monocyte, Macrophage, Scaffold
Resumo

O transplante de pele é de extrema importância para os pacientes que sofrem de perda severa de pele. Globalmente, o número de incidentes de queimaduras severas ao ponto de necessitar de tratamentos médicos ultrapassa o número de incidentes de tuberculose e VIH combinados. O transplante de pele por autoenxerto consiste em transferir pele de uma secção de uma área do corpo para outra, e apesar de suas limitações (necessidade de um procedimento cirúrgico adicional, disponibilidade limitada, e dor do local de extração), representa um método versátil e dinâmico de reconstrução cutânea, e continua a ser o tratamento padrão para lesões graves de pele. Embora tenham sido desenvolvidos e comercializados tratamentos regenerativos competentes, estes ainda apresentam limitações anatômicas, funcionais e estéticas. Esta dissertação está envolvida no desenvolvimento da camada dérmica de um substituto biossintético da pele: Skin2, mais concretamente, estudando a interação entre materiais candidatos a constituir este substituto dérmico com as células do sistema imunitário, nomeadamente a diferenciação destas células em contacto com os materiais e a degradação destes materiais por parte destas células.

Os monócitos são células do sistema imunitário que se diferenciam em macrófagos aquando do contacto com agentes patogénicos. Estes dois tipos de células estão entre as primeiras linhas de defesa contra corpos estranhos ao organismo, e desempenham um papel crucial na natureza da sua resposta a estes corpos estranhos, podendo induzir uma resposta inflamatória ou regenerativa. Sendo um enxerto de uma camada dérmica considerado um corpo estranho ao organismo, torna-se relevante estudar as interações entre os materiais candidatos a formar esta camada dérmica, e as células que determinam a natureza da resposta a estes materiais.

O objetivo desta tese foi estudar as interações entre macrófagos diferenciados a partir de monócitos (THP-1) e scaffolds celulares produzidos em laboratório por eletrofiação, nomeadamente: Viabilidade da linha celular monocítica THP-1, tal como a sua diferenciação em macrófagos e a polarização dos mesmos em fenótipo inflamatório ou regenerativo (M1 ou M2, respectivamente), quando em scaffolds produzidos com diferentes materiais e misturas desses materiais; Diferenças morfológicas dos macrófagos nos scaffolds, com diferentes materiais; A erosão gerada pelos macrófagos nos scaffolds.

Os materiais usados na eletrofiação foram dois polímeros de origem natural – Gelatina de pele de peixe de água fria (GEL), e Quitosano (CS) – e um polímero de origem sintética – poli-ε-caprolactona (PCL) – sendo cada um dos materiais usado individualmente ou sendo os materiais misturados entre si em misturas binárias ou ternárias. Os scaffolds produzidos foram depois reticulados com glutaraldeído (GTA) se tivessem CS ou GEL, de forma a manter as suas propriedades físicas, e cada um deles foi mais tarde semeado com células THP-1, em duas condições: com e sem adição de forbol-12-miristato-13-acetato (PMA), que induz a diferenciação de monócitos em macrófagos.

Numa primeira fase de cultura, foi semeada uma réplica de cada condição (cada scaffold e controles com e sem PMA) por sementeira, e foi verificada a viabilidade celular em cada material ao fim de 48 horas através do teste de resazurina. Estas sementeiras foram repetidas várias vezes, com a perspetiva de obter coerência de resultados com repetição do mesmo procedimento, solidificando quaisquer conclusões que pudessem ser retiradas dos mesmos. No entanto, esta coerência de resultados não se verificou, tendo-se verificado uma grande variabilidade nas viabilidades celulares para os mesmos materiais em sementeiras diferentes.
Devido ao facto de se terem gasto todos os materiais produzidos por eletrofiação nesta primeira fase, foi tentado um outro método de produção de materiais, teoricamente mais fácil e mais rápido: produção de filmes por evaporação de solvente. Os filmes a serem produzidos teriam, idealmente, a mesma composição química que os scaffolds de PCL, apenas alterando a morfologia em que os polímeros estavam dispostos. No entanto, a produção destes filmes tornou-se um desafio maior do que o previsto, tendo em conta que os filmes que continham PCL apresentavam uma clara separação de fases devido à imiscibilidade dos polímeros, o que fez com que fossem adicionados alguns compostos às soluções, como o Poly(óxido de etileno), visando torná-las mais viscosas, de forma a atenuar esta separação de fases, homogeneizando o filme. Esta tentativa de homogeneização não teve sucesso, pelo que a produção de filmes poliméricos foi abandonada e os materiais voltaram a ser produzidos por eletrofiação.

Foram ainda medidas as quantidades de endotoxinas em soluções poliméricas com os materiais utilizados em electrofiação, de forma a verificar quais destes continham valores de endotoxinas acima do limite médico (0,5 unidades de endotoxina por mililitro – EU/mL). Foi verificado que apenas a solução de GEL se encontrava acima deste limite, com 1,1 UE/mL na solução de concentração 1% (m/m) e com 1,5 EU/mL na solução de concentração 2%, enquanto as soluções de PCL e CS se encontravam dentro dos limites médicos, com a mesma concentração de 0,065 UE/mL. As endotoxinas foram removidas de GEL de duas formas: adição de peróxido de hidrogénio a uma solução de GEL que viria mais tarde a ser eletrofiada, e através do tratamento desidrotérmico (DHT) após a deposição da matriz por electrofiação. No entanto, a remoção das endotoxinas com a adiço de peróxido de hidrogénio impossibilitou a subsequente eletrofiação do material para produção do scaffold.

Seguiu-se então uma segunda fase de cultura, que passou a ser feita de acordo com um novo procedimento, sendo semeadas em cada sementeira 5 réplicas do mesmo material com PMA e 5 réplicas sem PMA, bem como controlos com e sem PMA, e sendo adicionado à cultura o material GEL com tratamento DHT. Após 48 horas, as células não aderidas foram retiradas dos poços e contadas, e foi feito o teste de resazurina com as células aderentes. Após este teste, foi adicionado meio de cultura aos poços, e o teste voltou a ser feito passadas 120 horas, de forma a verificar se a viabilidade se mantém ao longo do tempo.

Os novos resultados de viabilidade mostram que todos os materiais são viáveis, sendo que no scaffold de GEL que realizou tratamento desidrotérmico e no scaffold de PCL, há um aumento da viabilidade celular das 48 para 120 horas de cultura, sugerindo uma viabilidade prolongada nestes materiais, em contraste com os restantes, que apresentaram uma ligeira descida. Além disso, a estimativa das taxas de adesão mostra que scaffolds com misturas de polímeros tendem a promover maior adesão celular do que os scaffolds unitários.

Numa última fase, foi realizada microscopia de fluorescência com a finalidade de observar diferenças na morfologia celular dos macrófagos nos diferentes materiais, bem como na sua distribuição ao longo dos mesmos ao fim de 48 e 120 horas de cultura. No entanto, devido à elevada autofluorescência dos scaffolds de CS e de GEL no comprimento de onda de 555 nm (vermelho), a aquisição de imagens foi dificultada, bem como o seu tratamento. Apesar de ter sido possível obter imagens de microscopia de fluorescência, não são percutíveis quaisquer alterações morfológicas entre as células nos diferentes materiais.

Em relação à erosão gerada por macrófagos nos scaffolds, não foi observada qualquer degradação em microscopia eletrónica de varrimento (SEM). No entanto, os resultados do SEM possibilitaram uma observação mais clara da morfologia dos macrófagos e permitiram estimar o
seu fenótipo, de forma mais evidente do que com microscopia de fluorescência. A adição de PMA induz uma morfologia principalmente inflamatória (M1) nos macrófagos, com células arredondadas. Sem a adição de PMA, o fenótipo dominante aparenta ser o fenótipo inativado (M0) para os scaffolds unitários e para o scaffolds de CS GEL, e M1 para os scaffolds de CS PCL, GEL PCL, para a matriz ternária.

Palavras-chave: Polímeros, Eletrofação, Monócito, Macrófago, Scaffold
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Acronyms

CD – cluster of differentiation
CS – chitosan
DAPI – 4’,6-diamidino-2-phenylindole
DHT – dehydrothermal
EU – endotoxin units
FBR – foreign body reaction
GEL – gelatin
GTA – glutaraldehyde
IFN – interferon
IL – interleukin
iNOS – inducible nitric oxide synthase
LAL – limulus amebocyte lysate
LPS – lipopolysaccharide
MMP – metalloproteinase
MNGC – multinucleated giant cell
MW – molecular weight
PBS – phosphate-buffered saline
PCL – polycaprolactone
PFA – paraformaldehyde
PMA – phorbol-12-myristate-13-acetate
RPMI – roswell park memorial institute
SEM – scanning electron microscopy
Th1 – T helper type 1
Th2 – T helper type 2
TIMP – tissue inhibitor of metalloproteinases
UV – ultraviolet
1 Introduction

1.1 Context and motivation

Skin transplantation is of extreme importance for the survival of patients suffering from severe skin damage. In 2004, globally, there were more than 11 million incidents of burns that were severe enough to require medical attention, a number that is greater than the combined incidents of tuberculosis and HIV [1]. In many of these cases, the destruction of skin tissue may be so severe that there is a need for a skin graft. Even though there were significant improvements in patient and graft survival in the field of transplantation, grafting still presents limitations regarding donor sites and the risk of immunological rejection.

There are various treatment methods available for effective wound healing, resulting in low functional and cosmetic damage, such as epidermic substitutes or dermal substitutes, like Epicel®, or Integra®, respectively. However, these products have not yet achieved the same level of anatomical, aesthetic, and functional results as the autografts, which remain the standard option for treatment. Therefore, further investigation is needed to make advances in the efficiency of artificial skin grafts. Another relevant aspect of skin tissue engineering is that it opens doors for other parts of the body, while being easier to handle given its thin architecture. Recent statistics show that in July of 2019 there were more than 113,000 people on a waiting list for organ transplantation. More than 35,000 transplants have been done in 2018, but still, about 20 people die, daily, waiting for an organ transplantation (Figure 1.1) [2].

This dissertation is part of the Skin2 project, which aims to develop a biosynthetic bilayered skin substitute for the replacement of both dermis and epidermis, allowing for an improvement in extensive skin loss treatments. This biosynthetic skin substitute consists of biodegradable materials, which can be of biological or synthetic origin, and autologous cells.
Previous research has been made in this project concluding that two biological polymers – Cold water fish skin gelatin (GEL) and Chitosan (CS) – and a synthetic polymer – Polycaprolactone (PCL) – are good candidates for skin grafts, given that they promote healing of full thickness skin wounds in Wistar rats [3].

Studies regarding the optimization of the dermal scaffolds properties to properly support fibroblasts have been performed in our laboratory. The effect of electrospinning pore expansion or crosslinking of the dermal matrix have been tested to increase biological and mechanical properties of the dermal matrix. But other studies need to be performed, such as the interaction of the macrophages with the scaffold, given that those cells play an essential role in the acceptance or rejection of the grafts.

To do this, a human monocytic cell line derived from an acute monocytic leukemia patient named THP-1 was used. Cell viability was tested via resazurin assay, as well as cell adhesion rates to the dermal scaffolds, which allows inferring the macrophage activation rate. Macrophage generated erosion to the scaffolds was also evaluated via Scanning Electron Microscopy (SEM).

1.2 Objectives

The main goals of this project are:

- Produce the dermal scaffold using the electrospinning technique and various polymers.
- Study the THP-1 cells behavior in vitro upon contact with the scaffolds, namely, cell viability, the adhesion/differentiation rate, and morphological differences of macrophages under different conditions.
- Evaluate the effect of macrophages on the scaffold’s degradation.
2 Background

2.1 What is tissue engineering and regenerative medicine?

Tissue engineering refers to the scientific field concerned with the development of functional human tissue using the technologies of molecular and cell biology, as well as those of advanced material science and processing, in order to aid tissue regeneration when the human body is not likely to successfully regenerate itself [4]. It aims at attaining a full regeneration, by repairing or completely replacing tissues and organs that fail due to disease, genetic errors, congenital abnormalities, or traumatic injury.

Regenerative medicine is the field that includes tissue engineering, focusing on self-healing research, using the patient body mechanisms to trigger a regenerative response to foreign biological materials, being able to restore tissues and organs.

Tissue engineering relies on four main factors:

• The right scaffold – The scaffold is the structure that supports the cells in the tissue, presenting an architecture that is analog to the extracellular matrix. The goal of this structure is to help restoring tissue functions by temporarily supporting cell proliferation and extracellular matrix deposition. It may then be degraded, giving place to the extracellular matrix they helped to produce, or integrated in the tissue. Scaffolds can be derived from decellularized donor tissue or created in lab using natural or synthetic polymers, combined in a way to provide the intended mechanical and chemical behavior, adequate to the organ that is intended to be replaced. Different types of polymeric scaffolds (biodegradable porous scaffold, hydrogel scaffold, fibrous scaffold, ceramic scaffold and others) can be obtained using a variety of techniques (electrospinning, salt leaching, solvent casting, micro molding and others), having successfully integrated different types of tissues – bone, cartilage, ligaments, skin, neural tissues, etc [5]. The scaffolds may be degraded over time to integrate the host tissue without eliciting a string foreign body reaction.

• The right cells - The cells can be acquired from the target organ, developed from stem cells, or taken from lines developed in the laboratory. For clinical applications, the cells must be harvested from the patient to prevent rejection problems.

• The right biomolecules - The biomolecules may be added directly to the culture media or produced by the cells that are seeded in the scaffold, with the purpose of molding the microenvironment to be favorable for cells growth, migration and, finally, patient recovery. For example, vascular endothelial growth factor (VEGF), which has the role of initiating angiogenesis, when integrated in electrospinning scaffolds leads to and 80-fold cell migration increase, relative to a similar scaffold without VEGF. Additionally, it significantly enhances blood vessels formation in vivo [6]. In another study, interleukin 10 (IL10) was successfully bound to PCL electrospun fibers, with the ability to be absorbed over 14 days in vivo, inducing M2 (pro-regenerative) macrophage activation within the scaffold material and the surrounding tissue [7].
The right mechanical forces applied to the tissue – These forces should influence the activity of the cells as if they are in their natural environment, given that different cells from different tissues may have very different growth environments, and, for instance, a neuron should not grow in the same environment as a skeletal muscle cell.

The earliest clinical applications of tissue engineering were the creation of flat materials (essentially 2D), to aid in wound care. The first engineered tissues were, therefore, skin substitutes and some cartilage. In the late nineties, some cell culture sheets were produced ex vivo and applied to patients. It was relatively simple to culture these cells, given that the culture was in 2D and it was easy to deliver oxygen and nutrients to the cells. This fact led to the mistaken belief that the skin would be easy to replicate in vitro. Also, a large portion of Medicare – a health insurance company – money was being used to treat diabetes induced diseases, mainly diabetic ulcers, in the United States. Because of this, the skin tissue engineering became an attractive path, and it drew many efforts wound-care market [8].

Consequently, biodegradable scaffolds have been developed to improve healing in skin wounds. However, to date, a complete functional skin substitute is not available, and research is continuing to develop a competent full thickness skin substitute that can vascularize rapidly. The skin is among the first tissues to be subjected to an attempt of artificial creation.

Among many others, one essential aspect to consider is the use of materials and structures that limit rejection problems. Balanced monocyte and macrophage responses are essential for a successful immunological response, on which the success of the graft depends. Some tissue inflammation is essential for angiogenesis and stem cell activation, which later allows for tissue regeneration and healing. However, too much inflammation will result in rejection of the tissue [9]. Given that monocytes/macrophages play a crucial role regarding the nature of the immunological response (inflammatory vs regenerative), studying the interaction between these cells and biomaterials is particularly relevant for enhancing the efficiency of wound healing grafts.

2.2 Skin functions and anatomy

The skin is one of the largest organs of the human body. It is the physical barrier that separates the interior of the body from the external physical, biological, and chemical agents that may threaten its integrity. It is also essential for homeostatic, sensory, metabolic, and secretory functions, which are crucial for survival.

Skin is composed of two main layers: The epidermis and the dermis, which are separated by the basement membrane, right under the basal layer (Figure 2.1) [10]. Some authors consider the subcutaneous tissue as part of the skin, while others consider it as a separate layer on which the skin lies on, attaching it to the underlying bone and muscle, called hypodermis.
The epidermis is the outermost layer of the skin. It does not contain blood vessels or nerve endings. It is a layer composed mainly by cells called keratinocytes (about 90% of the cells), which are constantly proliferating and differentiating, in a process called keratinization, which is responsible by the epidermis auto renovation. Other cells present in this layer are the melanocytes (about 8%), responsible for skin pigmentation and UV radiation filtration, Langerhans cells, involved in immune responses, and Merkel cells, responsible for light touch sensation [11][12].

The dermis is a thicker layer that lies underneath the epidermis, and it is made of connective tissue containing nerve endings, blood vessels, and lymphatic vessels. This layer is composed mainly of extracellular matrix and fibroblasts, which are involved in the production of collagen, which is essential for the mechanical support of the skin [12].

The hypodermis is located beneath the dermis and is mainly composed of adipocytes [12].

2.2.1 Wound healing

Studying the process of skin wound healing is crucial for predicting the foreign body reaction (FBR) towards an implanted biomaterial for tissue repair and regeneration [13]. Skin wound healing is composed of three main phases: Inflammation, new tissue formation, and matrix formation and remodeling [12].

Inflammatory phase – is the initial phase of the injury (first four days). In this phase, coagulation is initiated, which cause a temporary layer of protection against foreign agents to the body, and it will prevent fluid loss. Afterwards, there is an increase in blood flow to that area, triggered by the inflammatory agents like histamine, which leads to the generation of a fibrin matrix, allowing for a temporary scaffold that supports cells migration and accomplishment of
micro vascularization [14]. Monocytes, macrophages, and other immunomodulatory cells are then attracted to the area of inflammation, as stated in the macrophages section.

Proliferation phase – occurs 5 to 20 days after injury, and it is characterized by the proliferation of fibroblasts and vascular endothelial cells by secretion of growth factors by inflammatory cells (mast cells, some macrophages, etc). Fibroblasts secrete collagen fibers that will replace the fibrin matrix overtime, and may differentiate into myofibroblasts, which have contractile abilities due to actin expression. This differentiation results in the contraction of tissue and closing of the wound. Adjacent healthy tissue initiates angiogenesis, which is followed by a migration of keratinocytes from the edges of the wound to the top of it, immediately below the blood clot.

Maturation phase – occurs from 20 days to months or even years after injury. This phase is characterized by the re-epithelization of the wounded tissue, and by the recovery of the dermis tensile strength.

Grafting technique is considered the golden standard for severe skin wounds. However, it still presents various limitations, such as availability of donor site, immune rejection, pain, scarring, slow healing and infection [15]. Therefore, a constant effort is being made to find an efficient way to overcome these limitations by developing tissue engineered skin, and these skin substitutes have been evolving exponentially. These can include materials composed of cells, extracellular matrix, or a combination of both.
2.3 Monocytes, macrophages and wound healing.

Monocytes are white blood cells that differentiate into macrophages upon contact with pathogenic agents. Monocytes derive from myeloblasts, which derive from the common myeloid progenitor cells, which, themselves differentiate from the multipotential hematopoietic stem cell. The common myeloid progenitor cells give origin to the myeloid branch, on which all blood cells are included, except for lymphocytes.

Monocytes and macrophages are among the first lines of defense against antigens. When there is a foreign body invasion, chemical signals are released in order to recruit blood clotting elements and phagocytic cells that will attempt to phagocyte them i.e., engulf them with their cell membrane and destroy them with hydrolytic enzymes from lysosomes (Figure 2.2a) [16]. Upon contact with the pathogens, the monocytes differentiate into macrophages, which release additional signaling molecules, named cytokines that cause capillaries to widen up and become more permeable, allowing for other phagocytic cells (neutrophils and more macrophages) to get to the injury site. These cells come to eliminate pathogens and cells debris, initiating tissue cleaning and healing. Sometimes macrophages cannot phagocyte larger pathogens or foreign bodies, so they merge into foreign body giant cells, and attempt to phagocyte them. When the foreign body is not completely engulfed, frustrated phagocytosis occurs (Figure 2.2b) [17].

![Diagram summarizing the process of phagocytosis](Image)

**Figure 2.2:** Left – (a): Diagram summarizing the process of phagocytosis; Right – (b): Types of phagocytosis. Normal, foreign body giant cell, and frustrated phagocytosis (left to right, respectively) [17].

Although it was believed for a long time that macrophages were originated exclusively from the differentiation of circulating monocytes, more recent studies show that these cells are present in mammals since the middle of gestation, contributing for physiologic homeostasis through life. These cells arise from yolk sack and liver progenitors, migrating to different tissues/organisms and persisting in those tissues/organisms as heterogenous, self-renewing tissue resident populations [18]. Tissue-resident macrophages are important for the fulfillment of tissue-specific and niche-specific functions, ranging from dedicated homeostatic functions, such as clearance of cellular debris and iron processing, to central roles in tissue immune surveillance, response to infection and resolution of inflammation [19]. Bone marrow-derived blood monocytes may also be recruited and differentiated after birth to replenish tissue resident macrophages populations and to give answer to increasing immunological demands upon an immunological response, such as inflammations or infections.
2.4 Cell communication and macrophage differentiation.

Cytokines are a type of small proteins (peptides) used for cell signaling [20]. They may be secreted by different cells, aside from immune cells like macrophages, mast cells, and B and T lymphocytes, endothelial cells, fibroblasts and stromal cells. Cytokines affect almost all biological processes, and play an essential role not only in immunoregulation, but also in the autocrine, paracrine, and endocrine systems. There are different types of cytokines: chemotactic cytokines (chemokines), interleukins (ILs), interferons, tumor necrosis factors (TNFs), and lymphokines.

Chemokines – This kind of cytokines’ function is to induce chemotaxis, i.e., induce movement of an organism in response to chemical stimulus. The main role of chemokines is to recruit leukocytes. However, angiogenic properties and collagen synthesis by fibroblasts have also been reported due to chemokines effects [21].

Interleukins – A type of cytokine that was first seen to be functional between leukocytes (hence the name, “inter-leuk”). Therefore, interleukins were believed to function only as modulators of immune functions. Although they are particularly important for this role, it is now known that the role of interleukins play a much greater role in the body than was initially though, regulating cell growth, differentiation and motility [22].

Interferons – Type of cytokines produced by the body cells as a defense against viruses. They are important modulators of the immune response. They can also combat bacterial and parasitic functions, inhibit cell division, and promote or impede the differentiation of cells. Interferons can be of type 1, which includes the forms – IFN-α and IFN-β – or they can be of type two, which includes the form IFN-γ. The difference of these types is that type 1 can be produced by almost types of cells upon contact with a virus and its goal is to increase their viral resistance, while type 2 interferons are produced by natural killer cells and T lymphocytes and aim to signal the immune system to respond to infectious agents of cancerous growth [23].

TNF’s – There are two types of TNF’s – TNF-α and TNF-β. TNF-α is mostly secreted by macrophages and it aims to kill tumoral cells. Additionally, it may stimulate the inflammatory response. TNF-β is produced by cytotoxic T-lymphocytes and aims to induce apoptosis in tumoral cells.
Monocytes and macrophages play a decisive role in the inflammatory response. Macrophages can differentiate into a spectrum of phenotypes, which range from pro-inflammatory (M1) to a pro-regenerative (M2a). There are different intermediate sub-phenotypes (M0, M2b, M2c), and each phenotype plays different roles in immunological response and wound healing (Figure 2.3) [17].

![Figure 2.3: Macrophage differentiation spectrum and respective precursors for their activation. It’s possible to observe that classical inflammatory macrophages, M1, are on the opposite end of the spectrum of wound healing macrophages, M2a [17].](image)

M1 – Pro inflammatory, kills intracellular pathogens by iron restriction and performs phagocytosis. They are also responsible for angiogenesis initiation, and recruitment of Th1 lymphocytes. This phenotype tends to assume a rounded shape [24].

M0 (also designed as M(-)) – Inactivated macrophages. These cells are differentiated from monocytes but have yet to polarize into a phenotype.

M2a – Anti-inflammatory, parasitic immunity and allergic responses. This cell phenotype tends to assume an elongated shape [24].

M2b – immuno regulatory, mixed phenotype, both pro-inflammatory and anti-inflammatory functions.

M2c – Plastic capacity, pro-healing function, such as matrix deposition (tissue remodeling).

Given this spectrum of phenotypes with different properties, it is wise to study how, exactly, macrophages react to biomaterial cues in order to increase grafts efficiency. It is important to know how the chemical and physical structure of biomaterials used in implants affects monocyte and macrophage response and differentiation in order to achieve the design of biomaterials with the intended properties, ideally, for any given situation. In this case, the goal is to achieve pro-regenerative properties and low inflammatory tendencies.

The interaction between macrophages and other cells is extremely complex and due to an environment that is influenced by uncountable factors. For example, an increased number of M1 differentiation will lead to the production of IFN- γ, and pro-inflammatory interleukins. IFN- γ leads to Th1 cells recruitment (T helper lymphocytes). These cells may then secrete TNF-α which will lead to more M1 differentiation, initiating a positive feedback loop for these biomolecules.
Conversely, the effect of some cytokines may be attenuated by the presence of others. For example, TNF-α and IL-12 expression are attenuated by IL-4. Even different balances of immune cells differentiation leads to different effects of each cytokine [25].

There is a complex feedback system between the macrophages and the adjacent cells in the tissue, like fibroblasts or mesenchymal stem cells. Each cytokine secretion stimulates different types of cells that will secrete other cytokines or chemokines, so, each cytokine may have different effects on the immune response depending on the environment of the wound, and the cells surrounding it. This is part of the reason why it is so difficult to perform a realistic study in vitro. There are many variables to consider, and in vitro models are often a very poor approximation of what happens in vivo.

2.5 Effect of endotoxins

Endotoxins, also known as LPS (lipopolysaccharides) are one of the most common pyrogens that induce a febrile reaction [26] [27]. This is a tiny, detectable, but hard to avoid type of contaminant that compromises many biocompatibility studies. These molecules are an integral part of the outer cell membrane of Gram-negative bacteria [28]. The most prominent LPS-sensitive cell population are cells of the monocyte/macrophage’s lineage. These cells produce a large variety of bioactive protein mediators in response to LPS, such as IL-1, IL-6, IL-8, and TNF-α, in order to defend the organism against potentially pathogenic agents. The massive release of cytokines, however, becomes hazardous for the organism by causing shock, cell damage, and multi-organ failure. Thus, the overproduction of those protein mediators leads to the manifestation of septicemia, multiorgan failure, and lethal septic shock syndrome [29].

Endotoxins are composed of an inner, hydrophobic lipid component – Lipid A – a core oligosaccharide, and an outer long heteropolysaccharide chain – O-specific chain – representing the surface antigen (O-antigen) (Figure 2.4) [30].

Endotoxins are very stable molecules, being able to resist to severe conditions, such as UV radiation, extreme pH, and temperatures up to 180°C. These molecules are insoluble in both methanol and ethanol. Therefore, they remain present after most sterilization procedures, compromising the biocompatibility studies.

Endotoxins are continuously released from bacteria during cell growth, division, and death. A single Escherichia coli cell contains about 2 million LPS. Since bacteria can grow in extreme conditions, they exist practically everywhere, including the air, tap water, or people’s skin. Hence, endotoxin contamination is very likely to occur upon incorrect handling of materials, especially when working with glass or plastic, given that their hydrophobic properties strongly promote endotoxin adhesion [28]. Even distillation and deionization processes cannot remove endotoxins from water. Chemical reagents, especially biological derived products, such as GEL or CS are also potential sources of endotoxins. For the purpose of decreasing endotoxin contamination probability, materials and reagents should be operated under a septic environment or laminar flow hoods. Exposure to air must be minimal. Such strict requirements are often challenging to meet.
The most well-known methods of endotoxin quantification are the rabbit pyrogen test (RPT) and limulus amoebocyte lysate (LAL), both approved by the FDA.

RPT consists of measuring the rise of temperature in rabbits that were given an injection of a test solution. This method is the oldest technique, it is not quantitative, and it does not give us the concentration of endotoxins. The LAL, conversely, is based on the aqueous extract of amoebocytes from the horseshoe crab, and it has three variant techniques: the gel-clot, the turbidimetric, and the chromogenic. The gel-clot technique is based on the coagulation of the LAL when in contact with endotoxins. The turbidimetric technique is based on the turbidity increase upon contact with endotoxins. The chromogenic technique is the most sensitive one. This technique uses a pre-clotting enzyme to hydrolyze a chromogenic substrate, which turns the test solution into yellow. The endotoxin concentration is related to the time required for the solution to turn yellow. By measuring the absorbances of solutions with known endotoxin concentration, it is possible to set a standard curve relating endotoxin concentration and absorbance. The sample is then subjected to the same procedure, the absorbance value is related to the standard curve, and the endotoxin concentration is found.

Endotoxins can be inactivated by either physical or chemical processes. A widely used example of a physical process is the dry heat method, which inactivates the endotoxins by incineration. This method is the established method of depyrogenation within the pharmaceutical industry [31]. The materials may be subjected to 250 °C for 30 minutes, 200 °C for 60 minutes, or 180 °C for at least three hours to achieve an efficient depyrogenation. Although this method is easy to use, not all materials can be subjected to such temperatures.

Another used method is the acid or alkaline hydrolysis, which consists of breaking the LPS molecules, more specifically, separating the lipid A from the oligosaccharide chain, resulting in reduced endotoxin activity. This method may be achieved by placing the material in a 1% glacial acetic acid from 2 to 3 hours, or in 0.05N HCl solution at 100 °C for 30 mins (both acid hydrolysis). As an example of alkaline hydrolysis, a NaOH solution may be used instead. The
time the material must be submerged to achieve depyrogenation depends on the concentration of
the solution.

Hydrogen peroxide has also been shown to be effective inactivating endotoxins under
reasonable temperatures while being safe to handle. However, this method may alter the
properties of the material that is being depyrogenated. 5% hydrogen peroxide solutions have been
shown to accomplish a total endotoxin inactivation for initial concentrations up to 1 EU/mL.
2.6 Scaffold production - Electrospinning

The electrospinning technique is used to obtain fibers from a polymeric solution. It is the most widely used technique to obtain fibers for application in tissue engineering. The electrospun fibers can be tuned to present the desired pore distribution, high surface area to volume ratio and cell adhesion motifs, due to their structural resemblance to the natural extracellular matrix [32]. This technique consists in introducing a polymeric solution in a syringe with a blunt tip needle, connected to a high voltage power supply and pointed at a grounded collector. The polymer in the solution will then be charged and subjected to an electric field. As a result, when the electrostatic repulsion forces become stronger than the surface tension of the polymeric solution, the drop of polymer in the needle tip stretches into fibers that will emerge out of it in the direction of the grounded collector (Figure 2.5) [33].

The morphology of the resulting fibers depends on deposition processing parameters such as:

- The voltage applied between the needle tip and the collector: as the voltage is higher, the polymeric solution is highly charged and the fibers are strongly stretched, due to self-repulsive forces, which results in higher fly speed of the solution towards the collector. Thus, with an increase in the applied voltage, a decrease in the resulting fibers diameter is observed. [34] [35]
- The feed rate of the solution through the syringe: The fiber diameter increases with the solution feed rate. An increased feed rate may lead to the formation of beads along the fibers due to the lack of solvent evaporation. [34] [35]
- The distance between the needle tip and the collector: It is intuitive to misbelieve that an increased distance leads to thinner fibers, due to a longer flight time which implies more time for fiber stretching. However, larger distances decrease the intensity of the electric field, compromising the stretching of the fibers. Therefore, using larger distances between the needle tip and the collector usually results fibers with larger diameters. Conversely, small distances usually result in the formation of fused fibers because the solvent does not evaporate completely and the fibers merge together upon contact. [34] [35]

Polymeric solution characteristics are also a factor to take into account in order to change fiber morphology, such as:

- Polymer concentration: An increased solution concentration leads to higher solution viscosity and resulted in fibers with increased diameter.
- Solution conductivity: Higher solution conductivity may be achieved by adding salt to the solution, leading to higher effect of the electric field on the fibers, which results in stretched and thinner fibers.
- Surface tension of the solution: Higher surface tension leads the conversion of the jet into one or several droplets minimizing surface area of the solution (Rayleigh Instability). This effect is countered by electrostatic repulsion between charges on the jet [35].
Environmental factors such as temperature and humidity influence the fibers morphology too.

2.7 Polymers used for electrospinning

The polymers to be used in this work are two of natural origin – chitosan and fish gelatin – and one of synthetic origin, polycaprolactone.

2.7.1 Chitosan (CS)

Chitosan is a linear, semi-crystalline polysaccharide composed of (1 → 4)-2-acetamido-2-deoxy-β-D-glucan (N-acetyl D-glucosamine) and (1 → 4)-2-amino-2-deoxy-β-D-glucan (D-glucosamine) units (Figure 2.6). Although it is not extensively abundant in nature, it can be easily obtained from partial deacetylation of chitin, the second most abundant polymer in the world, with cellulose being the first [36]. Chitin can easily be obtained from the exoskeletons of crustaceans and insects, or from the cell walls of fungi. The threshold that separates chitin from chitosan is not well established. Most sources refer that for the polymer to be considered chitosan, the deacetylation degree must be of at least 60%. However, some sources assume that chitosan deacetylation degree may vary from 35% to 95% [37], or 50% to 90% [38]. Its molecular weight ranges from 300 to 1000 kDA. Chitosan is usually insoluble in water, but easily soluble in weak acids (pH<6) due to the protonation of the amine group.

Chitosan is a promising polymer in tissue engineering due to its multiple biological properties, such as anti-microbial, anti-tumoral, and immune promoting properties [39].
The cationic nature of CS is responsible for electrostatic interactions with glycosaminoglycans, which are linked to numerous cytokines. CS has been shown to have a stimulatory effect on macrophages [37].

2.7.2 Gelatin (GEL)

Gelatin from cold-water fish skin (GEL) – A non-immunogenic protein obtained from partial hydrolysis of collagen, the structural protein of the dermal extracellular matrix.

Gelatin can be obtained from hydrolysis of collagen, the most abundant protein component of skin, tendons, connective tissue, cartilage and bones [40]. The hydrolysis can be achieved by high acidity or alkalinity, enzymatic effects, or high temperatures. Gelatin can be obtained from mammals or from fish, having different constitutions. The gelatin used in this dissertation is gelatin from cold water fish skin, which, unlike collagen, is soluble in water at room temperature. The structure of fish gelatin is largely composed of multiple repetitions of “Glycine-X-Y” sequences, where X and Y are proline and hydroxyproline, respectively (Figure 2.7) [40]. In mammals derived gelatin, there is a higher percentage of proline and hydroxyproline (about 30%) than in fish gelatin (17 to 25%), being that fish from cold water have lower percentage of these amino acids than fish from warm water, hence the higher solubility. For this reason, solutions made with mammals’ gelatin behave like a gel, and gelatin from cold water fish skin solutions behave like viscous liquids. In order to serve as a scaffolding material, gelatin polymeric chains have to be crosslinked either chemically, with glutaraldehyde (GTA) vapors or genipin, or physically, by UV radiation exposure or dehydrothermal treatment.

![Figure 2.6: Structure of the chitosan polymer chain. On the left it is represented the acetylglucosamine unit, and on the right the glucosamine unit (deacetylated) [36].](image1.png)

![Figure 2.7: Proline and Hydroxyproline](image2.png)
Upon hydrolysis, gelatin retains an important feature of collagen: it keeps the Arg-Gly-Asp (RGD)–like sequences of amino-acids, which promotes cell adhesion, migration and proliferation (Figures 2.8 and 2.9) [41].

### 2.7.3 Polycaprolactone (PCL)

Poly-ε-caprolactone is a biodegradable hydrophobic polyester characterized by high plasticity, ductility and slow degradation rate. This polymer is obtained by opening the monomeric epsilon-caprolactone cyclic ring, or by polycondensation of an hydrocarboxylic acid (Figure 2.10). Poly-ε-caprolactone is a semicrystalline polymer, whose degree of crystallinity may reach 69%, with a vitreous transition temperature of ~60 °C and a melting point of around 60 °C. The average molecular weight of this polymer varies between 3 and 100 kDA, and crystallinity tends to diminish with increased average molecular weight. PCL dissolves in organic solvents such as acetic acid, chloroform and dichloromethane, and precipitates in water. Its elastic properties make it easy to handle upon scaffold production techniques such as electrospinning, which makes it a strong candidate among synthetic polymers for tissue engineering.

![Figure 2.10: Structure of ε-caprolactone and Poly-ε-caprolactone upon ring opening, forming the polymer.](image)

However, its hydrophobicity is highly associated with weak affinity for cell adhesion. In order to surpass this problem, PCL tends to be mixed with biologically active polymers, resulting in a material with enhanced mechanical properties from PCL with the biocompatible advantages of other polymers.
2.7.4 Poly(Ethylene Oxide)

Poly(Ethylene Oxide) (PEO) is a linear polyether. It is a homopolymer obtained by catalytic polymerization of ethylene oxide, forming a linear neutral chain, with the formula \((\text{CH}_2\text{OCH}_2)_n\). It is nontoxic, biocompatible, electrochemically neutral and soluble in water and in many organic solvents. The oxygen atoms in its structure attract water molecules, making it hydrophilic. PEO can be used to aid chitosan electrospinning. In acid solutions CS positive charges originate electrostatic repulsions between chains, reducing their entanglement. PEO ether groups can interact with chitosan amine groups, diminishing those repulsions and facilitating the entanglement of the chains, resulting in an easier electrospinning. It is important to note, however, that PEO is soluble in water, and because of this it may compromise scaffolds stability/resistance to dissolution if used in large quantities [42] [43].

2.7.5 Crosslinking

Crosslinking allows for the manipulation of mechanical and structural properties of produced scaffolds, as well as their degradation rate or solubility. This is essential for GEL scaffolds due to their high solubility in water. Crosslinking is valued in tissue engineering by preserving scaffolds structural integrity both \textit{in vitro} and \textit{in vivo}. Polymer crosslinking is based on covalent bonds formed during the crosslinking process, between chains. Crosslinking methods can be chemical or physical. A common chemical method is by using glutaraldehyde (GTA), and the most common physical crosslinking process is dehydrothermal treatment (DHT). Both of these methods were used in this dissertation.

2.7.5.1 Glutaraldehyde

Chemical crosslinking mechanisms involve functional groups that allow bonding between polymeric chains and non-adjacent molecules. Although chemically crosslinked scaffolds tend to present better mechanical properties than physically crosslinked ones, they also usually present toxic effects [42]. The chemical crosslinking mechanism with GTA is presented in Figure 2.11 [44].

![GTA structure](image)

\textit{Figure 2.11: GTA structure (top); Mechanism of GTA crosslinking method: GTA forms long polymer chains that react with amine groups to form cross-links [44].}
2.7.5.2  Dehydrothermal Treatment Technique:

The DHT technique consists of subjecting the material to higher temperatures in vacuum, which leads to condensation reactions forming intermolecular crosslinks, as shown in Figure 2.12 [44].

![Figure 2.12: Mechanism of DHT crosslinking method: Carboxylic acid and amine groups form a crosslink through a condensation reaction [44].](image)

Besides being a non-toxic procedure, this technique also has the advantage of providing biomaterial sterilization by the high temperatures and long exposure periods to high temperatures.
3 State-of-the-Art

3.1 Scaffold’s characteristics effects on macrophages

To this day, not much is known about what exact biomaterials properties drives macrophages into M1 or M2 differentiation. Studies conducted with monocytes and macrophages show that macrophages change their behavior over time when in contact with different scaffolds, and make clear that not only chemical, but also physical factors influence this differentiation and behavior [24].

There are very few studies regarding macrophage response to GEL scaffolds, and none using gelatin from cold water fish skin. However, there are some studies using gelatins from other origins.

In the study performed by Wu et al. [45], the effect of an electrospun GEL matrix on macrophage modulation was evaluated, in comparison with a GEL film. They found that the number of macrophages adhering on GEL nanofibrous matrix was twice higher than the number of macrophages adhering on a GEL film. Additionally, macrophages in the film presented a predominantly inflammatory (M1) phenotype and, morphologically, they were less round and larger, in contrast to a non-polarized (M0) phenotype in the matrix.

Regarding PCL, Cao et al. [46] found that in vitro, monocyte adhesion rates decrease significantly in PCL aligned fibers, compared to random fiber depositions. They also found that in vivo, aligned fibers generated a thicker fibrous capsule when compared to the random fibers, and films generated a significantly thicker fibrous capsule than both aligned and random fiber scaffolds (37.7, 7.5, and 4.1 μm, respectively). Aligned fibers also presented higher cell infiltration.

Another study by J.R.Potas et al. [7] found that by placing IL-10 infused PCL electrospun scaffolds in vivo around peripheral sciatic nerves of Wistar rats, this interleukin was able to be released over a period of 14 days, resulting in M2 polarization within the scaffold material and in the adjacent tissue.

Wissing et al. [47] shows that scaffold microstructure strongly impacts macrophage response. In their study, they produced poly-ε-caprolactone-bisurea (PCL-BU) electrospun scaffolds. From a group of mats with 2 and 6 μm diameter electrospun fibers disposed in both isotropic and anisotropic ways, it was verified that mats with 6 μm fibers disposed in an anisotropic way suffered higher degradation by macrophages, which resulted in stronger erosion of the scaffolds. However, the differences in the degradation rate induced by macrophages could not be attributed to M1 or M2 polarization. Gene expression analysis showed that for macrophages seeded on anisotropic thicker fibers, both inflammatory and anti-inflammatory genes presented higher levels of expression than in the other conditions.

As for CS, Oliveira et al. [48] found that macrophages polarized towards M2c (pro-regenerative) phenotype, when in contact with CS films for 10 days. The levels of pro-inflammatory cytokines (IL-6, TNFα and IL-1β) decreased over time and anti-inflammatory cytokines (IL-10) increased.

Studies of 3D chitosan scaffolds grafted into mice showed that different degrees of acetylation resulted in different differentiation patterns. Macrophages seeded in scaffolds of CS with 5% DA possessed a much more regenerative macrophage phenotype (for F4/80+ and
CD206+) and cytokine secretion pattern (more IL-4 and less IL-6 and TNF-α) than macrophages seeded on scaffolds of CS with 15% acetylation, which present more inflammatory (F4/80+/CCR7+) factors [49].

Furthermore, in another study, chitosan molecular weight has been shown to have a strong impact on macrophage differentiation. Chitosan degraded to molecular weights of 152, 72, 7.1 and 3.3 kDa was seeded with RAW264.7 macrophages. It was observed that for larger chitosan molecular weight (300, 156 and 72 kDa), nitric oxide (NO) production – which is an indicator of inflammatory phenotype – was significantly inhibited and for smaller CS molecular weight, NO production was increased, indicating a more inflammatory phenotype for the smaller molecular weights. [39].

In a study by McWhorter et al [50], it was hypothesized that, since different macrophage phenotypes present different shapes, it would be possible to lead to a specific phenotype expression without exogenous cytokines by controlling cell shape. It has been shown that M1 macrophages presented a dramatically different shape from M2, being M1 rounded cells and M2 elongated cells. Micropatterning was then used to limit macrophages to 20 μm and 50 μm wide lines, forcing them to assume an elongated form. The results were compared along with non-patterned surfaces. It was shown that the elongated pattern cues led to M2 macrophage expression, and not only reduced the secretion of inflammatory cytokines, but also enhanced the effects of M2-inducing cytokines, IL-4 and IL-10 while limited the effects of M1-inducing stimuli LPS and IFN-γ. This study also concluded that by inhibiting actin-myosin contractility, the shape-induced polarization is negated, which suggests that the cytoskeleton plays a crucial role in macrophage polarization by cell geometry; although cytokine derived stimulation was not affected. Therefore, the architecture of the environment around the macrophages plays a heavy role in the outcome of their differentiation.

Failed biomaterial degradation attempts by macrophages has been linked to frustrated phagocytosis. Therefore, it was hypothesized that higher crosslinking densities would lead to more frustrated phagocytosis and, consequently, affect the severity of the foreign body response (FBR), given that a greater crosslinking density leads to higher resistance to degradation. However, when testing hydrogels crosslinked with different concentrations of glutaraldehyde (GTA) in vivo over three weeks, it has been shown that the FBR capsule thickness was not affected by the GTA crosslinking density. The M1 marker, inducible nitric oxide synthase (iNOS), remained consistent over the three weeks, and the M2 markers, Arg1 and CD163 increased overtime and were linked to FBR capsule thickness [51].

3.2 Macrophage generated matrix degradation

Depending on the macrophage phenotype, these cells secrete a variety of metalloproteinases (MMPs) or tissue inhibitor of metalloproteinases (TIMPs). MMPs degrade the extra-cellular matrix (ECM), and TIMPs inhibit MMPs and facilitate tissue regeneration [52], [53]. Annamalai et al. found that M1 macrophage phenotype tends to be correlated with a higher MMP/TIMP expression ratio when compared with M2 phenotype, on which TIMPs expression was much higher than in M1 phenotype [54]. Speidl et al also observed an MMP increase with inflammatory mediators [55]. Overall, studies show that M1 phenotype macrophages are much more responsible for matrix degradation than M2 phenotype macrophages.
Lysozyme has been shown to be involved in injury processes, and macrophages produce this enzyme [56]. Chitosan has been shown to be degraded by lysozyme depending on its DA, being that the higher the DA, the higher the degradation [57].

As for PCL degradation, the only found study was regarding the microarchitecture of the scaffold and it’s described in section 3.1 (Wissing et al) [47].

4 Material and methods

4.1 Solutions

Firstly, seven polymeric solutions were prepared for electrospinning. There were three unitary ones: gelatin (GEL), polycaprolactone (PCL), and chitosan (CS); three binary ones: a mixture of GEL and PCL, GEL and CS, CS and PCL; and a ternary one: a mixture of the three polymers. Poly (ethylene oxide) (PEO) was added to the solutions that contained CS, to allow the electrospinning process by increasing the viscosity of the solution.

GEL from cold water fish skin, PCL (Mw 80 kDa) and PEO (Mw 2 MDa) were bought from Sigma-Aldrich®. CS (Mw 500 kDa, deacetylation degree of 75.5%) was bought from Cognis®.

As for the solvents, ultrapure water was used along with glacial acetic acid ≥99.7% from Fisher Chemical.

The solutions were prepared in mass/mass accordingly to Table 4.1:

<table>
<thead>
<tr>
<th>Solution</th>
<th>Gel %</th>
<th>CS %</th>
<th>PCL %</th>
<th>PEO %</th>
<th>Acetic acid:Water %</th>
</tr>
</thead>
<tbody>
<tr>
<td>GEL</td>
<td>25</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>90:10</td>
</tr>
<tr>
<td>CS</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>0.4</td>
<td>90:10</td>
</tr>
<tr>
<td>PCL</td>
<td>-</td>
<td>-</td>
<td>20</td>
<td>-</td>
<td>100:0</td>
</tr>
<tr>
<td>CS GEL</td>
<td>2</td>
<td>2</td>
<td>-</td>
<td>0.2</td>
<td>90:10</td>
</tr>
<tr>
<td>CS PCL</td>
<td>-</td>
<td>2</td>
<td>2</td>
<td>0.3</td>
<td>90:10</td>
</tr>
<tr>
<td>GEL PCL</td>
<td>10</td>
<td>-</td>
<td>10</td>
<td>-</td>
<td>95:5</td>
</tr>
<tr>
<td>Ternary</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>0.2</td>
<td>90:10</td>
</tr>
</tbody>
</table>

Glass vials with screw lids were used to make the solutions. The correct amount of solvent and polymers indicated in Table 4.1 were weighted into the glass vials. The solutions were left in a “P-SELECTA® Agimatic – S” magnetic steering plate for 24 hours, at about 100 revolutions per minute.
4.2 Electrospinning

For the electrospinning, the solutions were loaded into 2mL sterile syringes (BRAUN Injekt® Luer Solo). Two syringes and two syringe pumps were used to allow for a faster deposition. 23G blunt needle tips are connected to the syringes which were placed on a syringe pump (KD scientific, KDS-100-CE). The pumps and the syringes stood outside the acrylic electrospinning cabinet, on opposite sides, to minimize electrostatic repulsion on the fibers, while only the needles went in through a circular opening with 1 cm diameter. The needle tips were then connected to a high voltage power supply (iseg T1CP 300 304p), through a cable and spinneret, and set at 18 kV. The cylindrical collector, with a slow rotational movement, was placed in between the two syringes, 25 cm apart from each other and covered with aluminum foil. The pumps were set for 0.3 mL/hour, for each of the solutions, and the depositions were performed for at least 3 hours, depending on if a satisfactory thickness was achieved. The thickness of the deposition may vary due to its spread on the aluminum foil, given that, for two depositions with the same deposition time, if one is spread through a wider area, it will necessarily be thinner.

The parameters were kept constant at 18 kV, 25 cm distance between needle tip and collector, and a flow rate of 0.3 mL/hour, except for situations in which it was visible that the deposition was not working, which may happen due to temperature variations, or, more likely humidity variations. In these cases, the parameters were varied by trial and error, as the fibers that were supposed to hit the collector were collected on a microscope slide instead, and observed under the microscope, allowing for the evaluation of fiber morphology and quality.

4.3 Crosslinking

For the glutaraldehyde (GTA) crosslinking, the fibrous mats that contained CS and/or GEL (all but the PCL unitary membrane) were placed in a hermetic box containing two vials of GTA from Merck, diluted to 50% concentration in water. The hermetic box was agitated for two and a half hours using an agitator inside the incubator at 37 ºC, allowing for GTA vapors to crosslink the GEL and CS polymers. After crosslinking, the depositions were placed into a vacuum chamber overnight to remove the excess of GTA from the scaffolds.

As for DHT crosslinking, it was only applied to a GEL scaffold, placing the scaffold in the incubator at 120 ºC for at least 4 hours.

4.4 Degradation tests

Degradation tests were performed for each type of scaffold, using lipase from Amano Enzyme Inc., Lysozyme from Fluka, Sigma Aldrich, and distilled water, and later also using hydrogen peroxide, from Carlo Erba Reagents, at 10% (v/v) and a buffer solution with a pH of 5.3, which is equivalent to the acidity of a 10% hydrogen peroxide solution at 25 ºC [58].

Lipase solution was prepared at a concentration of 0.1 mg/mL, for an activity of 10 U/mL, in distilled water, and the lysozyme solution was prepared at a concentration of 0.01 mg/mL in a phosphate buffer saline (PBS) solution with an ionic strength of 0.06 M and a pH of 7.4 to maximize the enzymatic activity (10 U/mL) [59]. For the second set of degradation tests, the hydrogen peroxide solution (10%) was obtained by diluting the stock solution (30%).
The 5.3 pH buffer was achieved by mixing the potassium dihydrogen phosphate at 0.1 M and the disodium hydrogen phosphate at 0.1M, in a 97.5 :2.5 ratio, respectively.

To each of the degradation solutions, 0.02% sodium azide from Merck was added to prevent contamination by gram negative bacteria.

As for the degradation tests, the procedure was the same for all solutions:

i. Three samples of fibrous mats of the same material, but from different electrospinning depositions were placed in vials and their initial mass was measured. The mass of the empty vials was also noted, and the initial mass of the material sample was obtained by subtracting the mass of the empty vials to the mass of the vials with samples.

ii. 10mL of distilled water was added to each vial in order to dissolve the soluble fraction of the matrices, over 24 hours in an orbital shaker.

iii. In the next day, the membranes were detached from the aluminum foil, which was removed, and the membranes were left to dry overnight in the incubator at 37 ºC.

iv. Both the aluminum foil and the vials with the membranes were weighted.

v. 10mL of degradation media were added to each of the vials and left for five days, in an incubator at 37 ºC under agitation.

vi. In the meantime, the degrading media was replaced every 48 hours to maintain the enzymatic activity close to constant. Next, the degrading media was removed and the vials with the matrices were washed with distilled water and left to dry at 37 ºC for 72 hours.

vii. The vials with the membranes were weighted. The degradation was evaluated over a period of 25 days.

4.5 Endotoxin quantification

Endotoxin quantification was made by following the Thermo Scientific™ Pierce™ Chromogenic Endotoxin Quant Kit protocol, which performs a chromogenic LAL test, as described in the endotoxins section.

This kit uses Ac-Ile-Glu-Ala-Arg-pNA as chromogenic substrate and 25% acetic acid as stop solution. Solutions of 25% acetic acid, 0.1M NaOH and 0.1M HCl (the last two guarantee a pH range from 6 to 8) were prepared using ultra-pure water as solvent. During the procedure, an oven was used at (37±1) ºC, and a thermal gel placed in a container made of expanded polystyrene to maintain the temperature. A 96-well microplate, where all standards and samples were placed, was used. The microplate was photometrically measured at 405nm by the absorbance microplate reader.

Four standard solutions were prepared with a known endotoxin concentration of 0.1, 0.25, 0.5, and 1 EU/mL. These solutions were prepared using the high standards provided by the kit to obtain a standard curve in the [0.1, 1.0] EU/mL interval. The kit also provided endotoxin-free water to use as a blank standard. Three replicates were used for each standard solution.
The sample preparation was made using 5 dilutions of materials with water: 2% PCL, 1% and 2% GEL, 1% and 2% CS, to compare the endotoxin contamination among the materials. Three replicates of each solution were prepared according to the following protocol:

- Prepare solution (m/v) of each material to be tested using ultra-pure water, accordingly to the desired concentration to be tested. This preparation is made in clean, chemically inert and closed containers that must be certified as non-pyrogenic.
- Leave solutions for 24 hours at 37 °C.
- Disinfect tubes with solutions, along with new tubes using ethanol 70% and bring them to the biological safety cabinet.
- Filter each solution and transfer it to the new tube using a disposable plastic syringe that is certified as non-pyrogenic, and sterile syringe filters of 0.22 µm.
- Adjust their pH for 6 to 8 and pre-equilibrate samples at room temperature before starting the endotoxin quantification assay as stated in the kit.

The equation for the standard curve has been determined by linear regression of the relative absorbance values at 405 nm, obtained from the standard endotoxin concentrations, using Microsoft Excel. This equation gives us absorbance (y) in function of the concentration of endotoxins (x) as \( y = mx + b \). Therefore, endotoxin concentration is calculated by a simple substitution:

\[
\begin{align*}
    x &= \frac{y - b}{m}
\end{align*}
\]

*Equation 1: Endotoxin concentration (x) in function of absorbance (y).*

**4.6 Endotoxin removal from gelatin**

The removal of Lipopolysaccharides (LPS) from the GEL was done by adding H\(_2\)O\(_2\) to the GEL solution, to the final concentration of 5% H\(_2\)O\(_2\), and placing it in a water bath for 2 hours at 90°C with the lid slightly unscrewed to prevent pressure accumulation. After this, the solution was placed in a heating plate at 72 to 80°C until all the solvent had evaporated. The resulting solute was then used to prepare solutions for electrospinning.

Another way of achieving GEL unitary matrix depyrogenation was through Dehydrotermal (DHT) treatment for four hours at 180°C. This process, however, was not possible for matrices containing PCL given its melting point at 60 °C, so, it could not be applied to either PCL+GEL scaffolds, or ternary scaffolds. Because of this, this method was only be applied to unitary GEL scaffolds.

**4.7 THP-1 Culture**

For cell culture assays, the cells used were THP-1 (ATCC® TIB-202™). These cells were cultured in a vertically placed T-25 flasks at 37 °C and 5% CO2 in a Sanyo®MCO-19AIC(UV)
CO₂ Incubator using RPMI 1640 culture medium from Biowest, completed with 10% heat inactivated fetal bovine serum (FBS, Biowest), 1% penicillin/streptomycin (Invitrogen), 1% L-glutamine, 1% sodium pyruvate 100 mM (100x) (Gibco), 1% non-essential amino acids (100x) (Gibco). The cell concentration was kept between 200 k and 1 M cells/mL, with the culture medium being changed every 48 to 72 hours.

4.8 THP-1 Culture on the materials

A protocol for cell seeding on the fibrous scaffolds was established:

Day 1: Fibrous mats cutting, sterilization, and detachment from the aluminum foil:

- Three samples of each type of deposition were cut with a hole puncher, with 12mm diameter, and placed into Petri dishes.
- The samples were sterilized with 70% ethanol.
- As the membranes became soft and malleable, they were detached from the aluminum foil using two sets of tweezers; The aluminum foil was discarded and the petri dishes with the matrices were left on the flow chamber to dry overnight.

Day 2: GTA Neutralization with glycine:

- Filtered glycine (1% concentration m/m) was added to the matrices to neutralize the GTA, and the dishes were closed and left overnight.

Day 3: Cell seeding:

- The glycine was removed, and the matrices were washed with RPMI.
- The matrices were lifted using a 12 mm lamella and two sets of tweezers and placed in the in house made Teflon inserts with 0.5cm² area which were placed in a 24-well plate, with two repetitions for each material.
- Macrophages were seeded at a density of 80 × 10⁴ cells/cm² over a 0.5cm² area on each sample. Empty wells of the 24 well plate were seeded with the same cell density and used as controls.
- Introduction of 12-O-Tetradecanoylphorbol-13-acetate (PMA), from Sigma-Aldrich at a 25 nM final concentration in the wells of one of the samples of each material, in order to promote macrophage differentiation (based on [60]):
  - Take 10 µL of the stock PMA solution, with a concentration of 1 mg/mL, with is equivalent to 1.6mM (M_{PMA}=616.83 g/mol).
  - Mix with 990 µL of culture medium and divide into ten vials and freeze for future use.
  - Of one of the vials, take 5.2 µL and mix with 295 µL of medium.
  - Pipette 20 µL of the resulting dilution to half of the wells with inserts, and 80 µL to the ones without; the remaining wells got the same amount of culture medium to keep an equal cell concentration.

Day 5: Resazurin Viability Assay:
• Resazurin (from Alfa Aesar) solution, prepared in PBS at a concentration of 0.04g/L, was added to each one of the wells in the same volume of medium presented there. Resazurin solution with culture media were dispensed in wells without cells to be used as reference.
• The plate was incubated at 37 °C and 5% CO2 for two hours.
• After incubation, medium absorbance was measured in a BioTek® ELx800 spectrophotometer, and the absorbance was read using Gen 5™ Microplate Reader and Imager Software, from BioTek®, at 570 nm and 600 nm. The correct absorbance was obtained by subtracting the absorbance measured at 600 nm to the one measured at 570 nm and subtracting the reference medium. This value is proportional to the cell viability.

4.8.1 Reformulation of the cell culture protocol

Each cell culture was now performed with a single material, with 5 replicas for each condition (culture with PMA and without PMA). 12 mm glass coverslips were placed in Teflon inserts and used as cell controls. Sterilization, GTA neutralization with glycine, and cell seeding were done in the same way as described in the previous section. However, the PMA concentration was changed to 8 nM, and GEL DHT was added as a culture material. The procedure of the 5th day was adapted as follows:

• The culture medium of each well was collected into Eppendorfs, along with non-adherent cells.
• Each of the wells was then washed twice with PBS, which was also added to the respective Eppendorf.
• The Eppendorfs were then centrifuged for 5 mins at 200G’s in a Centurion Scientific K3 Series, with a BRK5424 rotor.
• The supernatant was carefully discarded, and the number of cells was counted using Trypan Blue.
  o To the cells attached to the matrices, a solution containing 50% resazurin stock solution (0.04 g/L in PBS) and 50% culture medium (final concentration of 0.02 g/L)) was added and the culture was placed in the incubator at 37 °C and 5% CO2 during 4 h.
• After the 4 hours, the absorbance was read, the same way as previously explained in the previous “Cell Culture” section.
• The resazurin viability assay was measured again after 72 hours.

4.8.1.1 Estimation of the number of adherent cells

• Calculate the cell proliferation rate by dividing the concentration (in cells per mL) of cells in the non-PMA control well by 200k (initial concentration);
• Divide the concentration of cells in each well by their proliferation rate to get the number of cells in suspension at the beginning of the culture.
• The initial concentration of adherent cells is equal to 200k minus the number of cells in suspension (assuming they will not proliferate).
• The number of cells adhered or in suspension, in each well, can be directly obtained from their concentration by dividing it by 5 (since only 1/5th of a mL was added to the well);
• Calculate the average of the number of cells in suspension and adhered for the wells with and without PMA, and the rates of adhesion.

4.9 Film production by solvent casting

Because electrospinning matrices take long to produce, films were produced from the solutions prepared according to Table 4.2, with the goal of evaluating the chemical effect of the polymers on the cell viability without the influence of the scaffold’s structure. The solutions were poured into 86 mm diameter glass Petri dishes and left to dry at 37ºC in the incubator for 24 hours.

Table 4.2: Initial polymer and solvent concentration in each of the films produced.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Gel %</th>
<th>CS %</th>
<th>PCL %</th>
<th>PEO %</th>
<th>Acetic acid:Water %</th>
</tr>
</thead>
<tbody>
<tr>
<td>GEL</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0:100</td>
</tr>
<tr>
<td>CS</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>2:98</td>
</tr>
<tr>
<td>PCL</td>
<td>-</td>
<td>-</td>
<td>5</td>
<td>-</td>
<td>100:0</td>
</tr>
<tr>
<td>CS GEL</td>
<td>2</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>2:98</td>
</tr>
<tr>
<td>CS PCL</td>
<td>-</td>
<td>2</td>
<td>2</td>
<td>-</td>
<td>90:10</td>
</tr>
<tr>
<td>GEL PCL</td>
<td>2</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>90:10</td>
</tr>
<tr>
<td>Ternary</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>-</td>
<td>90:10</td>
</tr>
</tbody>
</table>

As for the films containing PCL mixed with other materials, there was a clear phase separation. Solution viscosity was therefore increased to avoid the molecular migration and aggregation, which results in a phase separation in the films.

To increase the viscosity of GEL PCL solutions, the polymers concentration was increased from 2% to 10%. Additionally, PEO with molecular weights of 900 kDa, 400 kDa, and 2 MDa was added to the solutions in different concentrations (each to its own solution), as a trial and error, to test if any of the solutions would provide the intended results. The solutions are presented in Table 4.3.

Table 4.3: Concentration (%wt) of PEO in the GEL PCL solutions for film production, by molecular weight. Each entry corresponds to a solution (4 solutions in total).

<table>
<thead>
<tr>
<th>Solution</th>
<th>PCL</th>
<th>GEL</th>
<th>PEO 400k</th>
<th>PEO 900k</th>
<th>PEO 2M</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10%</td>
<td>10%</td>
<td>2%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>10%</td>
<td>10%</td>
<td>4%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>10%</td>
<td>10%</td>
<td>-</td>
<td>2%</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>10%</td>
<td>10%</td>
<td>-</td>
<td>-</td>
<td>1%</td>
</tr>
</tbody>
</table>
In another attempt to increase solution viscosity, GTA was diluted in acetic acid to a final concentration of 2% (v/v). Either 0.5 mL or 1 mL of this solution were added to the 10 g of PCL/GEL solution for films production, which was agitated for 1 min in the steering plate. This was the time at which the solution could mix the best, right before it turned into a semi-solid gel. At this point, the solution was spread in a petri dish and put in the incubator it at 37ºC until all the solvent was evaporated. This method allowed the simultaneous crosslinking of GEL.

As a last attempt to prevent phase separation, the solutions were evaporated while sealed in hermetic boxes with 2 containers that had GTA 50% for 6 to 16 hours.

4.10 Scanning Electron Microscopy (SEM)

Electrospinning membranes were prepared for observation in a scanning electron microscopy (SEM) from Hitachi TM3030 Plus by cutting small pieces and placing them in double sided carbon tape with the matrix side up. The other side on the tape was stuck to a circular metal plate. Before the observation of the samples, they were sputter coated – coated with a layer of about 10nm of a conductive material, in this case, a mixture of gold/palladium (60/40), in order to increase signal to noise ratio during SEM imaging and therefore produce higher quality images. The SEM was operated in high vacuum at 15 kV.

4.11 Nanofiber diameter quantification (image J)

The diameter of the electrospun fibers was measured using the imageJ software and histograms of the fiber diameter distribution were plotted in Excel. For each SEM image with a magnification of 3000x, at least one hundred measurements of fiber diameters were made from different parts of each picture.

4.12 Fluorescence microscopy

Two cell cultures were prepared according to the second cell culture protocol. 48 and 120 hours after seeding the cells, cell fixation and staining with DAPI, Helix Green, and phalloidin 555 and 488 CruzFluor® took place.

Cell Fixation Procedure:

- Remove culture medium from the well.
- Wash three times with PBS++ (PBS containing Mg$^{2+}$ and Ca$^{2+}$).
- Add paraformaldehyde (PFA) solution (4%) for 10 to 15 minutes (200uL/well).
- Wash three times with PBS.
- Leave in PBS at 4ºC until staining.

Staining with phalloidin and DAPI:
• Wash with PBS.
• Add 200 µL of triton X-100 0.5% for 10 minutes.
• Wash with PBS 3 times.
• Add 100 µL of 5% DAPI, 0.1% phalloidin and 0.2% Helix Green in BSA and leave for 30 minutes.
• Wash with PBS 3 times.
• Wash with ultrapure water 3 times.
• Take membranes of wells and put them on 20uL of mounting media in coverslips, with the cells facing the mounting media.
• Leave in the dark.

After the staining was done, the cells were viewed under a Nikon ECLIPSE Ti microscope and at least two images were taken for each of the conditions at 100X and 400X magnification.

The second observed culture had different staining agents added to it depending on the material. Either combination A – Helix Green 6 and phalloidin 555 – or combination B – DAPI and phalloidin 488 – were added to each material. Combination A was added to PCL, GEL DHT and control, and combination B was added to CS, GEL, CS PCL, GEL PCL, GEL CS and MT.

4.13 Fluorescence microscopy image processing (ImageJ)

The images acquired through Fluorescence microscopy were analyzed using the ImageJ software. Every image was converted into 32bits images and subjected to a background removal and a contrast/brightness adjustment, with the parameters variating according to necessity in each image. The color channels were then merged, and the final images were created.

4.14 Macrophage generated Scaffold erosion

Another cell seeding was created with the 8 materials, with and without the effect of PMA, according to standard “Cell Seeding” protocol. The only difference was that 100k cells were seeded in each well instead of 40k, in order to amplify the results of degradation. A control seeding was also created containing only the materials and culture medium. Both cultures were left at 37ºC for 120 hours.

After this period, the medium and was removed, and the “cell fixation” protocol, from section “Fluorescence microscopy” was followed. Control culture was also submitted to cell fixation protocol to maintain consistency.

The scaffolds were then dehydrated by ethanol gradient: PBS was removed from the wells and a succession of ethanol solutions were added in increasing concentration. Each solution was left in the well for 10 minutes. The concentrations used were: 30%, 50%, 70%, 80%, 90%, 100%, in this order.

The scaffolds were then left to dry and were prepared for SEM according to the protocol of the “SEM” section.
5 Results and discussion

5.1 Fiber morphology

SEM images of the fibrous scaffolds were analyzed using imageJ. Fiber morphology was analyzed, and fiber diameters were measured. One hundred measurements were made for each image, and the images are shown in Figure 5.1, along with the respective histogram of the fiber diameters, and the average results and standard deviations are presented in Table 5.1.

<table>
<thead>
<tr>
<th></th>
<th>Average measurement (µm)</th>
<th>Standard Deviation (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GEL</td>
<td>0.67</td>
<td>0.12</td>
</tr>
<tr>
<td>CS</td>
<td>0.43</td>
<td>0.12</td>
</tr>
<tr>
<td>PCL thin (&lt; than 0.7 µm)</td>
<td>0.34</td>
<td>0.07</td>
</tr>
<tr>
<td>PCL thick (&gt; than 0.7 µm)</td>
<td>1.26</td>
<td>0.29</td>
</tr>
<tr>
<td>GEL PCL</td>
<td>0.51</td>
<td>0.25</td>
</tr>
<tr>
<td>CS GEL</td>
<td>0.49</td>
<td>0.11</td>
</tr>
<tr>
<td>CS PCL</td>
<td>0.53</td>
<td>0.12</td>
</tr>
<tr>
<td>MT</td>
<td>0.69</td>
<td>0.18</td>
</tr>
</tbody>
</table>
As expected, results showed that the different materials presented some differences in fiber morphology for the same conditions of deposition.

Gel fibers merged with each other forming a net, instead of a set of fibers stacked on each other. This happens because of GTA crosslinking. Each individual fiber was flattened, unlike the other materials, which present cylindrical shape fibers.

CS fibers showed the thinnest average fiber diameter with the fibers having the narrower fiber diameter distribution. Some fibers tended to merge forming thick sets of fibers.

PCL fibers have not only the higher average fiber diameter, but also the highest standard deviation, containing both thick fibers and thin fibers, and almost no fibers with average diameters.

In CS GEL fibers, the average fiber diameter is higher than in CS scaffolds and lower than in GEL scaffolds. Overall fiber morphology is similar to the CS scaffolds, with a less visible effect of crosslinking on the fiber’s morphology relative to GEL.

GEL PCL had a large quantity of very thin fibers. There is a reduction in average fiber diameter compared to GEL and PCL unitary scaffolds. It seems that the presence of GEL did not allow for the formation of the thick PCL fibers, and the PCL presence did not allow for an effective crosslinking in GEL, not allowing for the fibers to achieve a flattened morphology, which also results in a lower apparent diameter.

For CS PCL scaffolds, the fibers had a large average fiber diameter when compared to CS and a thinner average fiber diameter than in PCL. There is a high number of merging aligned fibers which may be interpreted as a single fiber.

As for the Ternary Mix, most of the fiber presented diameters between 0.65 and 0.85 µm, which is the highest average value after PCL. The fibers were the ones with the lowest standard deviation.

5.2 Degradation tests

The performed degradation tests with lysozyme, lipase, hydrogen peroxide and buffer with pH 5.3 are presented next.
5.2.1 Lysozyme and lipase

It was possible to observe that the scaffolds degradation varies depending on the polymer and degrading agent. PCL, for instance, clearly degraded faster in lipase when compared to other degrading solutions. This is visible not only on the PCL degradation graph (Figure 5.2), but also in the binary scaffolds containing PCL, where the mass decreased by half after 20 days of degradation and in the ternary scaffold which lost about one third of its mass after the same period, corresponding to the proportion of PCL in the matrix. As for the other membranes, no significant enzyme activity seems to be present regarding both enzymes. Being a glycoside hydrolase that catalyzed the hydrolysis of 1,4-β-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in peptidoglycan [61], it was hypothesized that lysozyme would be able to degrade chitosan, but that was not the case in this study, as further bibliographic research reinforced [62], [63]. The results for lipase and lysozyme (and ultra-pure water as control) are shown in the following graphs:

![Graphs showing the degradation of scaffolds with different enzymes and solutions.](image-url)
5.2.2 Hydrogen peroxide and equivalent pH buffer solution

As for degradation studies regarding the effect of H$_2$O$_2$, there was a much faster degradation of both CS and GEL scaffolds, although PCL scaffolds were also affected. After just 5 days, the remains of GEL and CS scaffolds were barely visible, and as 10 days went by, these were completely degraded (Figure 5.3). However, pH 5.3 buffer did not affect the scaffolds, just like distilled water, which implies that the degradation of the scaffolds was not due to hydrogen peroxide’s equivalent pH, but instead due to the action of reactive oxygen species.

It is worth noting that there was an apparent mass increase along this study for scaffolds. This is probably because very small masses were being measured, and the effect of external factors such as temperature and humidity highly influence the results of the weightings. Even the temperature decrease that occurred after taking the vials out of the incubator resulted in a mass gain overtime due to pressure decrease derived from temperature decrease, and entrance of air molecules into the vials to balance inside and outside pressures (approximately 2-5 mg increase.

![Graphs showing degradation over time for different scaffolds.](image)

*Figure 5.2: Matrices mass loss over 25 days for different degrading agents: Distilled water (control), Lysozyme, and Lipase; for each of the seven materials used in electrospinning: GEL (a), CS (b), PCL (c), GEL and PCL (d), Gel and CS (e), CS and PCL (f), and Ternary Mix (g). Results presented in percentage of mass relatively to the initial mass. Lysozyme effect showed in blue, Lipase in orange, and distilled water in gray.*
from the start of the weighting of the 63 vials, to the end). However, this experiment still provides valuable information for the effect of each degradation agent on each material.

Figure 5.3: Unitary Scaffolds degradation in contact with different degrading agents: Distilled water (control), hydrogen peroxide, a saline buffer with pH 5.3, for the three unitary scaffolds – GEL (a) PCL (b) and CS (c) – and for the ternary scaffold (d). Graphs show remaining mass, relative to initial mass. Hydrogen peroxide induced mass loss shown in blue, buffer in orange and distilled water in gray.
5.3 Film production

Not all the films produced had the desired characteristics. For the unitary material films, the production was successful (Figure 5.4). However, for the binary films containing PCL and for the ternary film, it was possible to observe a clear phase separation (Figure 5.5). This implicates that the films containing PCL with the other polymer (GEL and/or CS) would not be suitable for cell culture because the seeded cells would not be in contact with a mixture of materials, but instead, some cells would be in contact with PCL and others with GEL/CS.

When PEO was added to the solutions the morphology of the films has not changed and phase separation was still observed.

![Figure 5.4: Unitary Films successful production. CS (left), GEL (middle), PCL (right).](image)

![Figure 5.5: CS PCL phase separation (left); Ternary mix phase separation (right)](image)

The addition of GTA (2%) to the solutions was not a successful method to produce uniform films either. Either 0.5 or 1 mL of GTA 2% solution were added to the 10 g polymeric solution, with the resulting solution being mixed for the maximum amount of time before it gained a hydro-gel consistency, which would make it impossible to produce the films. Solutions were then poured into petri-dishes. Even at such high viscosity limit, the resulting films still present phase separation (Figure 5.6, left). On very high concentration solutions, like GEL 10%,
PCL 10%, PEO (400 kDa) 2%, with 0.75 mL of GTA 2% added, the film contracted and became rubbery (Figure 5.6, right).

With the solvent casting technique in a hermetically sealed box containing GTA 50%, it would be possible to produce a more uniform film. However, the polymers that were subjected to this method always acquired a rubbery consistency (Figure 5.7), probably due to material contraction. Such severe chemical and mechanical properties alterations were not intended. Hence, such material would not be a valid candidate for cell culture, not only because it had lost the initial intended properties, but also because it was not comparable to the materials produced by electrospinning.

The main goal of this method, which was to produce more materials at a quicker pace to be used in cell culture was unsuccessful.
5.4 Endotoxin quantification

The standard curve of the wave absorbance at 405nm was obtained for the known endotoxin concentrations of 0.1, 0.25, 0.5 and 1 EU/mL (Figure 5.8). A linear tendency is visible and described by the equation next to the graph, along with the respective coefficient of determination ($R^2$).

![Standard Curve](image)

*Figure 5.8: Standard Curve of optical absorbance in function of endotoxin concentration obtained in chromogenic LAL test, used for calculation of endotoxin concentration in GEL 1% and GEL 2% solutions. Each of the points in the graph corresponds to the average of three replicates. The linear tendency equation is presented next to the graph along with its coefficient of determination ($R^2$).*

The kit specification stated that standard curves must have a coefficient of determination that is higher or equal to 0.98 for the LAL test to be reliable. This condition was verified, and the standard curve is indeed able to be used for determining the endotoxin of the solutions in aim.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Relative absorbance (405nm)</th>
<th>Calculated EU/mL</th>
<th>Within Medical Limit Threshold?</th>
</tr>
</thead>
<tbody>
<tr>
<td>2% PCL</td>
<td>$6.7 \times 10^{-4}$</td>
<td>0.065</td>
<td>Yes</td>
</tr>
<tr>
<td>1% CS</td>
<td>$-3.3 \times 10^{-4}$</td>
<td>0.065</td>
<td>Yes</td>
</tr>
<tr>
<td>2% CS</td>
<td>$3.3 \times 10^{-4}$</td>
<td>0.065</td>
<td>Yes</td>
</tr>
<tr>
<td>1% GEL</td>
<td>1.7</td>
<td>1.1</td>
<td>No</td>
</tr>
<tr>
<td>2% GEL</td>
<td>2.3</td>
<td>1.5</td>
<td>No</td>
</tr>
</tbody>
</table>

*Table 5.2: Calculated endotoxin concentration of the polymeric solutions, along with absorbance values.*

Endotoxin concentrations calculated out of the interval $[0.1, 1]$ EU/mL may be inaccurate due to extrapolation. However, it was possible to determine that PCL and CS solutions have endotoxin concentrations well within the medical limit (0.5 EU/mL), while none of the GEL solutions did – Table 5.2.
5.5 Endotoxin removal with hydrogen peroxide

The method described in “Endotoxin Removal from Gelatin” resulted in a foamy substance that was used to create endotoxin-free GEL solutions to be used in electrospinning. However, none of the various solutions made using this method were successfully electrospun. Instead, these solutions appeared to be sprayed along the collector. For this reason, it was hypothesized that there were severe chemical alterations in the GEL that broke polymeric chain. To confirm or deny this, both the original GEL and the one that resulted from the endotoxin removal procedure were submitted to Fourier-Transform Infrared Spectroscopy (FTIR).

Even though this method significantly alters GEL structure, it’s been shown that it reduces endotoxin concentration to under the medical limit, decreasing concentration to 0.38EU/mL down from 1.7 [64].

Gelatin and depyrogenated gelatin were observed under FTIR to observe if there were significant structural changes in the material (Figure 5.9).

Peaks of 3300-3250 cm⁻¹, 1630 cm⁻¹, 1530 cm⁻¹, 1395 cm⁻¹ and 1245 cm⁻¹ are present in both types of GEL, and correspond to Amide A (N-H stretch), Amide I (mainly C=O stretch), Amide II (N-H bend in plane and C-N stretch), alcohol (O-H bending) and Amide III (N-H bend in plane and C-N stretch) groups, respectively [65] (Figure 5.9, green).

![Figure 5.9: GEL and Depyrogenated GEL FTIR results. Matching peaks marked in green, unmatching peaks marked in red.](image-url)
The appearance of peaks in depyrogenated GEL in the 3660 cm⁻¹ (O-H stretching, free alcohol), 2990-2900 cm⁻¹ (C-H stretching, alkanes), and 1066 cm⁻¹ (O-H stretching, primary alcohol) [66] show significant changes in the polymer structure (Figure 5.9, red).

5.6 Cell viability with first protocol

Results on cell viability at this time of application of this protocol was highly inconsistent. For this reason, the same experiment was repeated several times, with the aim of acquiring more consistent results. However, such results were never achieved with this protocol. Some of those results may be found in the “Additional Content” section.

The inconsistency might have happened for a variety of reasons, including:

- Experimental mistakes – Inexperienced handling of the electrospun matrices generated a lot of variability, especially when the membranes were very thin. Many factors, such as air temperature, medium pH, partial evaporation of ethanol, different concentrations of glycine, keep adding variability to this experiment.
- Wrong PMA concentration – The concentration of PMA used in this protocol might not have been the appropriate. After further bibliographic research, it turned out that 8nM was the best concentration of PMA for both cellular adhesion and CD14 expression (macrophage indicator) [67].
- Endotoxin contaminations of polymers – The scaffolds were not produced using GEL with an endotoxin content below the accepted for medical grade use, which adds a lot of variability to the experiments.

Despite these inconsistencies, it was possible to conclude that all the polymers are viable for THP-1 culture, at least on short term.

5.7 Reformulated protocol

5.7.1 Resazurin assay

The macrophage population on each material with the reformulated protocol is represented in Figure 5.10 (directly proportional to Absorbance):
Results show that with PMA, the macrophage population is always higher than without PMA, as expected, since the non-adherent cells were washed away after 48 hours.

GEL PMA is the material with most macrophage population. However, the population in the control PMA condition of this experiment is also much higher than in other experiments, indicating better culture conditions, which may be the cause for such a high population. This type of gelatin presents a higher macrophage population than GEL DHT in the first 48 hours. However,
GEL DHT has an increase in population from 48 to 120 hours, unlike non treated GEL, with GEL DHT presenting a higher population than GEL after 120 hours for the condition without PMA.

The lowest population is seen on PCL without PMA after 48 hours. However, there is an increase of more than 100% from 48 hours to 120 hours of culture in this condition, indicating this is a viable material in the long term.

In the binary scaffolds and ternary scaffolds, the viability differences between the conditions with and without PMA tend to be softened, compared to the unitary scaffolds, especially in the first 48 hours.
5.7.2 Adhesion rates

Figure 5.11: THP-1 adhesion rates in each material with (+) and without (-) PMA in Chitosan (a); Polyaprolactone (b); Gelatin from cold water fish skin (c); Gelatin from cold water fish skin with dehydrothermal treatment (d); Chitosan and Gelatin (e); Chitosan and Polycaprolactone (f); Gelatin and Polycaprolactone (g); and Ternary Mix (h);
From the results depicted in Figure 5.11, when CS is mixed with other polymers, a substantial inhibition of the PMA effect was observed, which should make the cells adhere. For instance, both PCL and CS unitary scaffolds present considerable differences regarding cell adhesion rates when PMA is present and when it is not. These differences of adhesion between conditions reach 75% in CS and 90% in PCL unitary scaffolds. However, looking at CS PCL scaffold, the presence of PMA is not that significant, and the difference in adhesion rate lowers to about 25%. In GEL CS scaffolds, this inhibition is more evident, and the effect of PMA is barely noticeable. GEL PCL mix and the Ternary Mix also show reduced PMA effect relative to the unitary scaffolds, although not in such a big extent as binary scaffolds containing CS.

Another interesting observation is that GEL that underwent dehydrothermal treatment promoted higher adhesion rates than standard GEL, which seems counterintuitive, since standard GEL has endotoxin levels above medical stipulated. Higher quantities of endotoxins should, in theory, generate a more immediate response, leading to a higher monocyte differentiation and macrophage M1 activation rate, which is directly correlated to cell adhesion.

5.8 Fluorescence microscopy

At least two images were taken for each of the conditions, with different magnifications (x100 for observation of spatial distribution and x400 to analyze macrophage morphology). The results are displayed in the Figures 5.12 to 5.26:
<table>
<thead>
<tr>
<th>Control 48 hours</th>
<th>-PMA</th>
<th>+PMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>X100 magnification</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>X400 magnification</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
</tbody>
</table>

*Figure 5.12: Fluorescence microscopy of macrophages in the control condition after 48 hours of culture without and with PMA (left and right, respectively), with a magnification of 100 and 400 (top and bottom, respectively).*

<table>
<thead>
<tr>
<th>Control 120 hours</th>
<th>-PMA</th>
<th>+PMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>X100 magnification</td>
<td>![Image]</td>
<td>x</td>
</tr>
</tbody>
</table>

*Figure 5.13: Fluorescence microscopy of macrophages in the control condition after 120 hours of culture.*
In the control, glass coverslips without matrices, as expected, there was a clear difference in cell distribution depending on whether PMA is present or not. Not only there is a higher quantity of cells with PMA, but there are also many more aggregates. There are no significant differences between the images obtained after 48 hours of culture (Figure 5.12) and the image obtained after 120 hours of culture (Figure 5.13).

For CS, it was impossible to form a full, merged image due to the strong autofluorescence of CS, especially for 555 nm wavelength (red). However, looking at the images where the cells
were stained with helix green, the difference in cell distribution is clear when PMA is added to the culture (Figure 5.14). For the 120 hours culture (Figure 5.15), it was only possible to take images with PMA, since the other condition did not present any fluorescence. This might have been due to a mistake handling of samples during staining procedure, like not properly getting rid of PBS before applying the staining agents, which dilutes them. It is worth noting that green the staining agent on the 120 hours culture is Phalloidin 488, and blue stain is DAPI, unlike the previous images that had Helix green and Phalloidin 555 (red). The formation of clusters/aggregates is still clear.

![Figure 5.16 Fluorescence microscopy of macrophages in GEL after 48 hours of culture without and with PMA (left and right, respectively), with a magnification of 100 and 400 (top and bottom, respectively).](image1)

![Figure 5.17 Fluorescence microscopy of macrophages in the GEL after 120 hours of culture without and with PMA (left and right, respectively), with a magnification of 400.](image2)
For the 48 hours GEL culture (Figure 5.16), the GEL has high autofluorescence in the 555 nm wavelength, and complete images could not be constructed. However, observation of cells stained with helix green showed a difference in number of the cells when PMA is added to the culture, which results in more cells and more aggregates. With the staining of the nuclei with DAPI in the 120 hours culture (Figure 5.17), it was possible to obtain a more detailed image, compared to the 48 hours culture. It was observed a lower quantity of cells when PMA is added, judging by the quantity of nuclei. However, each nucleus with PMA measures about 1.5 to 2 times the diameter of the ones without PMA, and there is a much stronger Phalloidin expression per cell in the +PMA culture.

![Figure 5.18: Fluorescence microscopy of macrophages in gel DHT after 48 hours of culture without and with PMA (left and right, respectively), with a magnification of 100 and 400 (top and bottom, respectively).](image)
For GEL DHT, a decrease in autofluorescence for 555 nm wavelength was observed compared to standard GEL, making it possible to form merged images (Figures 5.18 and 5.19). No significant morphological differences were observed for the macrophages in the presence and in the absence of PMA. An increase in the number of cells was observed when PMA was added to the culture media. In the 120 hours culture, only the (-PMA) culture was observed. There seems to be an increase in cell concentration/quantity compared to the 48 hours culture. Additionally, the estimated cell adhesion rate is 33%, which is a large enough percentage to, with some mitosis, reach concentration levels like the ones shown in the 120 hours photo. These results are highly backed up by the viability results of this material, which had a 60% increase from 48 to 120 hours without PMA, and a 37.5% increase in the same time interval with PMA.
Figure 5.20: Fluorescence microscopy of macrophages PCL after 48 hours of culture without and with PMA (left and right, respectively), with a magnification of 100 and 400 (top two rows and bottom row, respectively). Second set of photos with x100 magnification aim to show the uneven distribution of cells in the sample.

Figure 5.21: Fluorescence microscopy of macrophages in gel PCL after 120 hours of culture without and with PMA (left and right, respectively), with a magnification of 400.
PCL matrices (Figures 5.20 and 5.21) presented a large heterogeneity in cell distribution. Both images with x100 magnification were taken from different parts of the same samples for each of the conditions. These images allow us to see deepest cell infiltration, when compared to other polymeric mats. There seems to be a much higher cell density on mats seeded without PMA after 120 hours, but the most probable cause for this is the heterogeneity in cell distribution in PCL as seen in the 48 hours culture pictures.

**Figure 5.22**: Fluorescence microscopy of macrophages in CS PCL after 48 hours of culture without and with PMA (left and right, respectively), with a magnification of 100 and 400 (top and bottom, respectively).

**Figure 5.23**: Fluorescence microscopy of macrophages in CS PCL after 120 hours of culture without and with PMA (left and right, respectively), with a magnification of 400.
The images of CS PCL samples reinforced the idea that CS attenuates the effect of PMA when mixed with other polymers, concerning cell adhesion, as predicted upon cell counting and adhesion rates calculation. In the first 48 hours there seems to be a higher quantity of cells in the +PMA condition (Figure 5.22). However, in the 120-hour culture, the quantity of cells in the -PMA condition appears to have exceeded the +PMA condition (Figure 5.23). Viability assays showed a higher decrease of cell viability in +PMA condition than in -PMA.

For GEL PCL (Figures 5.24 and 5.25), cells were not visible on samples without PMA, which should not happen, given that cell adhesion rates were almost 40%. These samples had a strong autofluorescence in 555 nm wavelength. From the 48 hours culture to 120 hours culture, there seems to be an increase in overall cell size and a decrease in the number of cells.
For the first culture on the ternary scaffolds (Figure 5.26), only the staining with helix green rendered perceptible images, where the nuclear distribution along the scaffolds were clearly observed. For the 120 hours seeding, it was not possible to obtain any information from the images.

CS GEL mats presented autofluorescence, which prevent the acquisition of pictures in any condition.

5.9 Macrophage generated erosion

This experiment aimed at the observation of matrix degradation generated by macrophages. The degradation of the samples was observed by SEM and the representative images are depicted in Figures 5.27 to 5.34. Such degradation was barely noticeable in all conditions for 5 days of culture. However, clear phenotype differences were visible amongst the macrophages in almost every material, between the culture containing PMA and the culture without PMA. Such differences were not so easily observable in fluorescence microscopy.
For PCL, some degradation was noticed when the cells were seeded without PMA, as the fibers seem corroded in the brightest areas, although it was not significant. In the absence or in the presence of PMA the cells clearly infiltrated in the PCL mats (Figure 5.27). As for macrophage morphology, the culture without PMA has mainly round cells with no cytoplasmic extensions, apparent of M0 phenotype. In the culture with PMA, the cells present a flattened shape, characteristic of M1 phenotype.

Figure 5.27: SEM analysis of PCL electrospun scaffolds at x1000 and x2000 magnification (left and right, respectively) without and with PMA, as well as a control condition (scaffolds that had been put into culture medium without cells).
In CS scaffolds (Figure 5.28), there are no visible fiber morphological alterations, indicating lack of degradation. Unlike PCL mats, CS mats had small pore sizes that prevented cell infiltration, being visible the cells only on the surface. Morphologically, the cells in culture without PMA presented a rounder shape than the cells cultured with PMA, which presented a mixture of phenotypes, having some round cells and some flat cells, but predominantly flat cells. This suggests that the predominant phenotype of cells seeded with PMA is M1/M0, and without PMA the phenotype is more polarized to M0. However, even though there isn’t as much M1 expression without PMA, there’s still a mixture of M1 and M0.

![Figure 5.28: SEM analysis of CS electrospun scaffolds at x1000 and x2000 magnification (left and right, respectively) without and with PMA, as well as a control condition (scaffolds that had been put into culture medium without cells).](image)
Upon observation of the GEL scaffolds (Figure 5.29), the samples with cells cultured with PMA were not successfully observed in SEM. There is no visible scaffold degradation. The analysis of the mats with cells cultured without PMA showed no cell infiltration, and some small cell clusters. Morphologically, the cells present a round shape, with some cytoplasmic extensions, although they are not very discernible. The main polarization seems to be M0 to M1, with a phenotype closer to M0 in the polarization spectrum than CS and PCL.

Figure 5.29: SEM analysis of GEL electrospun scaffolds at x1000 and x2000 magnification (left and right, respectively) without PMA, and a control condition (scaffolds that had been put into culture medium without cells). Pictures with PMA were not successfully acquired.
GEL DHT samples also presented low cell infiltration (Figure 5.30). No scaffold degradation was visible. Clusters of cells were observed in GEL DHT samples in the presence and in the absence of PMA, although in the culture with PMA larger clusters were visible. Both conditions presented the same main morphological characteristics, consisting of round cells with barely visible cytoplasmic extensions, like the normal GEL scaffolds. This is the only polymer in which the predominant morphology in culture with PMA is a round type of cell, which suggests a more neutral predominant phenotype than all the other observed polymers.

Figure 5.30: SEM analysis of GEL DHT electrospun scaffolds at x1000 and x2000 magnification (left and right, respectively) without and with PMA, as well as a control condition (scaffolds that had been put into culture medium without cells).
In Figure 5.3, no degradation was observable. Some cell infiltration was detectable on the mats with cells cultured with PMA. In this condition, the cells presented a flattened morphology with extensions towards the interior of the pores. On the culture without PMA, however, the cells were still with a round shape and larger enough to infiltrate in the pores. On the culture with PMA, cells presented an M1 phenotype morphological characteristics, while on the culture without PMA they presented M0 phenotype morphological characteristics.

Figure 5.3: SEM analysis of CS GEL electrospun scaffolds at x1000 and x2000 magnification (left and right, respectively) without and with PMA, as well as a control condition (scaffolds that had been put into culture medium without cells).
CS PCL scaffolds (Figure 5.32) presented low cell infiltration and no visible degradation. The predominant macrophage phenotype is M1, especially in culture without PMA, with some of these cells presenting an elongated shape. In the culture containing PMA, there was a mix of phenotypes, with a high number of cells with round shape, characteristic of M0, some round flat cells, characteristic of M1, and some elongated cells, characteristic of M2.

Figure 5.32: SEM analysis of CS PCL electrospun scaffolds at x1000 and x2000 magnification (left and right, respectively) without and with PMA, as well as a control condition (scaffolds that had been put into culture medium without cells)
For GEL PCL, cells in culture with PMA were not visible in the scaffolds. There is significant cell infiltration in the scaffold, on the culture without PMA, with all the visible cells as flat (M1 polarization).

Figure 5.33: SEM analysis of GEL PCL electrospun scaffolds at x1000 and x2000 magnification (left and right, respectively) without and with PMA, as well as a control condition (scaffolds that had been put into culture medium without cells).
Regarding the Ternary Mix, there was no visible scaffold degradation, and there was low cell infiltration. In both conditions, the predominant macrophage polarization is M1, with a flat morphology, with some cells presenting a rounded shape, characteristic of M0.

Figure 5.34: SEM analysis of Ternary Mix scaffolds at x1000 and x2000 magnification (left and right, respectively) without and with PMA, as well as a control condition (scaffolds that had been put into culture medium without cells).
6 Conclusions

The first goal of this project consisted of producing dermal scaffolds using electrospinning technique with several polymers, including unitary, binary, and ternary scaffolds. All the scaffolds were successfully produced using the electrospinning technique.

Creation of an electrospun scaffold with endotoxin-free gelatin was not possible, due to the chemical changes suffered by GEL upon endotoxin removal using hydrogen peroxide. Therefore, binary scaffolds containing GEL and ternary scaffold had endotoxin levels above the medical limit, which might have triggered an excessive inflammatory response, compromising the study. For unitary GEL scaffolds, it was possible to remove the endotoxins using the DHT method.

An additional mean of producing materials for cell culture – film production – was attempted with little success given the poor miscibility between PCL and the other two polymers. No films without phase separation were obtained.

Future studies could incorporate PLA with GEL and CS matrices to make a ternary scaffold from which endotoxins can easily be removed by DHT at 120°C, given that PLA has a melting point of 150 to 160°C, unlike PCL that melts at 60°C.

The second goal was to study THP-1 cells behavior in vitro upon contact with the scaffolds, namely, the macrophage population, adhesion rates, differentiation rates, and morphological differences of macrophages under different conditions. In every polymeric mat, the number of adherent cells – macrophages – increases when PMA was added, as expected, given that more monocytes differentiate into macrophages when PMA is present.

Another aspect taken into consideration comes from looking at PCL and GEL DHT cell number results, in which there was an increase in cell population from 48 to 120 hours of culture, both with and without PMA. Such an apparent increase in population may be explained by two reasons:

- The average metabolic activity of cells increased overtime due to macrophage polarization, resulting in a higher resazurin metabolic rate.
- The cells in these conditions kept multiplying on a higher rate than they died, resulting in a higher overall resazurin metabolic rate.

Given the heterogeneity of macrophage phenotypes on the PCL scaffolds upon SEM examination and given the fact that the population increased both with and without PMA for this scaffold, the second hypothesis is the most likely. Upon looking at GEL DHT SEM image, it was observed that the cells with and without PMA presented similar phenotypes, and the number of cells in the (+) PMA condition is much greater than in the (-) PMA condition. Since the population increased overtime for both types of observed predominant phenotypes, the hypothesis that the increase in cell viability is, in fact, explained by continued cell multiplication is reinforced.

Cell populations in the other materials either remained constant or slightly dropped from 48 to 120 hours, possibly due to a higher cell death rate than division rate.

Analysis of adhesion rates for each material show that polymer blends are inducing cell adhesion, especially blends containing CS, with adhesion rates higher than 60% without the influence of PMA in CS PCL, CS GEL, and Ternary Mix. There is a much higher adhesion rate
in these blends and also in GEL PCL than in any of the unitary scaffolds. This suggests an induction of monocyte to macrophage differentiation by these scaffolds.

Fluorescence microscopy imaging was performed with the goal of visualizing cell distribution across the materials and morphological differences in the macrophages. However, although it was possible to have an idea of cell distribution, no relevant morphological differences were observed using this technique. Nonetheless, analysis of SEM aimed at degradation test analysis provided valuable information regarding macrophage morphology, which made phenotype estimation possible. In all observed polymers, when PMA was present, macrophages were flat shaped and occupied a much larger surface area, which is characteristic of M1[68], except in GEL DHT, GEL (images were not successfully acquired), and GEL PCL with PMA (no cells were visible in this condition). In the cultures without PMA, the predominant phenotype seems to be M0, for unitary scaffolds and for CS GEL scaffold, and M1 for the CS PCL, GEL PCL, and Ternary Mix scaffolds.

Overall, PMA appears to have an inflammatory effect on the cultures, and the polymeric mats with the most apparent inflammatory inducing properties are CS PCL, GEL PCL, and Ternary Mix, given the predominance of flat cells.

It is important to notice there is a very significant viability variation in the control condition across the different experiments, meaning there was a relevant amount of variation between conditions or mistakes were made in preparations of the different assays. For this reason, future studies are required to acquire more consistent, reproductible results that solidify any conclusions that may be taken from these assays.

Regarding differentiation studies, PCR studies could be applied to analyze the molecules secreted by the macrophages, allowing for a more objective phenotype estimation.

As for the third goal, regarding scaffold degradation, the results were inconclusive, since there was no visible degradation in any of the scaffolds, except residual degradation of PCL. Further studies should be performed with a higher cell seeding density and longer culture times.
7 Bibliography


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8 Annex

8.1 Viability graphs of first cell culture protocol

Before reformulating the cell culture protocol, there were several attempts to get consistent results with the first protocol. Some of those results are presented in the following graphs:
Figure 8.1: Inconsistencies in THP-1 viability tests on different cultures using the original protocol. In each graph, there is the viability of each material with and without PMA, as well as the controls, after 48 hours of culture.