**Development of hyaluronic acid/plasma derived
fibrin hydrogels for the optimization of
dermo-epidermal skin substitutes**

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Dedicated to my family and friends, always pushing me to new heights

*"The success of a production,
depends on the attention paid to detail".*

David O Selznick

*"Details matter.
It's worth waiting to get it right".*

Steve Jobs

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1. Rey Legidos, J. M., Cubo Mateo, N., Montero Simon, A., Garcia Diez, M., Velasco Bayon, D., & Jorcano Noval, J. L. (2016). Development of a hyaluronic acid/plasma-derived fibrin hydrogel for the optimization of dermo-epidermal autologous equivalents. *Frontiers in Bioengineering and Biotechnology*, (743) DOI: <https://doi.org/10.3389/conf.FBIOE.2016.01.00743>
2. Montero, A., Quílez, C., Corral, A., Hernandez, R., Velasco, D. & Jorcano, JL. (2018). Cell-Free Mediated Contraction of Plasma-Derived Fibrin and Collagen Type I Hydrogels. *The International Journal of Artificial Organs*, 41(9):486–631. SAGE Publications Sage UK: London, England. DOI: <https://doi.org/10.1177/0391398818785526>
3. Montero, A., Valencia, L., Corrales, R., Jorcano, J. L., & Velasco, D. (2019). Smart Polymer Gels: Properties, Synthesis, and Applications. *In Smart Polymers and their Applications* (pp. 279-321). Elsevier. Woodhead Publishing. DOI: <https://doi.org/10.1016/B978-0-08-102416-4.00009-0>
4. Stojic, M., Lopez, V., Montero, A., Quílez, C., de Aranda Izuzquiza, G., Vojtova, L., Jorcano, J. L., & Velasco, D. (2019). Skin tissue engineering. *Biomaterials for Skin Repair and Regeneration* (pp. 59-99). Elsevier. Woodhead Publishing. DOI: <https://doi.org/10.1016/B978-0-08-102546-8.00003-0>

References to these publications appear throughout the entire thesis.

Abstract

Skin is one of the largest organs of the body and covers almost its entire surface. Its main functions are protecting the body against the hostile environment, regulating the body temperature, inhibiting water loss and also functioning as sensory organ. Skin damage thus is presented as a potential threat to the maintenance of the homeostasis between the body and its surroundings. Full-thickness wounds resulting from acute damage, severe burns and other chronic conditions like diabetic foot ulcer represent a huge medical burden due to the difficulty to treat them and the high cost, fact that will make the skin wound care market to reach a \$12.45 billion dollars market capitalization by 2022. Classical solutions to skin damage have been the use of autografts and allografts to transplant healthy skin parts into the wounded area. However, drawbacks associated to these techniques have rendered them of limited utility and the emergence of skin tissue engineering and more precisely of dermo-epidermal constructs has opened the possibility to fully treat these wounds in an effective manner. In this thesis, we use a solution previously developed in our laboratory consisting on a dermo-epidermal skin equivalent comprised of a plasma-derived fibrin dermal scaffold containing embedded human primary fibroblast and a layer of keratinocytes seeded on top to form the epidermis. Although this matrix has been proven to be effective for the treatment of severe and extensive burned patients in Spain, several persisting issues associated with this solution still remain unsolved. Furthermore, its use as an *in vitro* platform for disease modelling and drug testing has been hindered by its reduced stability. Here we perform a complete characterisation of the plasma-derived matrices and the effects of different concentrations and environments on them in terms of contractile behaviour and mechanical endurance, to later propose a solution to the issues related to them in the form of poor mechanical properties and excessive degradation rates. A new research trend proposes the combination of biological components and biomaterials with different properties to form enhanced scaffolds that are able to overcome the aforementioned issues. In that context, this thesis shows that hyaluronic acid (HA), a glycosaminoglycan naturally present in the skin, in combination with fibrin-based matrices is capable of better mimicking the wound healing environment, reducing the shrinking rates and enhancing the mechanical properties. HA at physiological levels (0.05%-0.2%) was used in

combination with a crosslinker polyethylene (glycol) diacrylate (PEGDA) to better reinforce the plasma-derived matrices. The results obtained for the preliminary characterisation studies were satisfactory in terms of mechanical and biological properties for a HA content of 0.05% w/v and 2:1 crosslinking ratio (moles of thiol to moles of acrylate). Organotypic skins formed *in vitro* with these conditions showed to be up to three times thicker than the plasma controls evidencing the reduction in contraction while they also showed a better and more homogeneous Keratin 10 (K10) expression in the supra-basal layer of the epidermis. Furthermore, filaggrin expression showed the formation of an enhanced stratum corneum for the constructs containing HA. The next step is transplanting this new dermo-epidermal equivalent into the backs of nude immunodeficient mice in order to check the integration and viability of the construct *in vivo*. If successful, this finding might open the possibility to use these matrices in patients in the future leading to improved outcomes and better handling for the surgeons. Furthermore, our findings can be useful for reducing the time and costs associated to the technique while also serving as a potential *in vitro* testing platform for the study and development of new pharmacological products.

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List of abbreviations

HA – hyaluronic acid
ECM – extracellular matrix
PEG – polyethylene glycol
PEGDA – polyethylene glycol diacrylate
TE – Tissue Engineering
GAG – glycosaminoglycans
US – United States
WHO – World Health Organization
EGF – epidermal growth factor
KGF – keratinocyte growth factor
TGF- β – transforming growth factor- β
VEGF- vascular endothelial growth factor
FGF – fibroblast growth factor
2D – two dimension
3D – three dimension
CEA – cultured epithelial autograft
PLGA – poly (lactic-co-glycolic) acid
POx – poly (oxazolines)
PVA – poly (vinyl alcohol)
PAA – poly (acrylic) acid
TF – tissue factor
FpA – fibrinopeptide A α
FpB – fibrinopeptide B β
RGD - Arginylglycylaspartic acid
HP – heparin
HS – heparin sulfate
CS – chondroitin sulfate
DS – dermatan sulfate
KS – keratin sulfate
PG – proteoglycan
MW – molecular weight
HAS – hyaluronic acid synthase
HYAL – hyaluronidases

CD44 – Cluster of Differentiation 44

RHAMM – Receptor for Hyaluronan Mediated Motility

ICAM-1 – Intercellular Adhesion Molecule 1

ISO – International Organization for Standardization

GMP – Good Manufacturing Practices

CE - Conformité Européenne

PBS – Phosphate buffered saline

FBS – fetal bovine serum

DMEM – Dubbelco Modified Eagle's Medium

P/S – penicillin/streptomycin

SEM – Scanning Electron Microscope

O.D. – Optical Density

Introduction

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1.1 Human skin

1.1.1 Introduction

The skin is the outermost part of the human body, covering the whole surface with an approximate size of 1.5 to 2 square meters depending on the individual [1]. It comprises around the 15% of the human weight, making it one of the largest organs in the body. The main functions of the skin are the protection of the body from external environment, including pathogens, radiation etc.; thermoregulation of the body through sweat glands and a lipid layer; the maintenance of the hydration and the production of vitamin D among other functions [2, 3].

Structurally, the skin can be divided into three main components: the epidermis, the dermis and the hypodermis. Each of these structures differs from each other in composition and function and work together in maintaining the overall homeostasis inside the body [4].

1.1.2 Skin anatomy

Anatomically speaking, the skin has been profoundly studied since ancestral times. As stated before, three different anatomical compartments comprise the human skin: epidermis, dermis and hypodermis. Figure 1.1 resumes the main parts that make up the skin.

Two main types of skin have been described in the literature, the thick skin with epidermis thickness ranging between 0.8 and 1.5 mm and thin skin, with the thickness ranging between 0.07 and 0.15 mm [6]. Thick skin is found in both the palmar and plantar surfaces of the body and lacks hair follicles as well as sebaceous glands [7]. On the other hand, thin skin is found elsewhere and lacks in its composition the stratum lucidum layer found in thick skin [6, 8].

Epidermis

The epidermis is the upper layer facing the exterior environment. It is a keratinised structure formed mainly by a single type of cell, the keratinocytes [8]. Depending on the type of skin, this layer is comprised of different internal lay-

Figure 1.1: Skin structure. The outermost stratum is the epidermis, a stratified layer of keratinocytes. Underneath it is found the dermis, which consists of dense irregular connective tissue and cushions the body from stress and strain. Finally the hypodermis is the innermost layer of the skin, mainly used for fat storage [5].

ers better known as strata. From the inside to the outside, these strata are the stratum basale, stratum spinosum, stratum granulosum and stratum corneum (Figure 1.2). In the case of thick skin an additional stratum called stratum lucidum sits between the stratum corneum and the stratum granulosum [9].

Epidermis is constantly renewing itself with the cells at the stratum basale, some of them stem cells, constantly dividing to give rise to new basal and suprabasal keratinocytes generating new skin every four weeks [6, 11]. Other types of cells present in the epidermis are the melanocytes, Merkel cells and Langerhans cells [12].

Melanocytes form part of the basal layer of cells, comprising around 5-10% of the cell population and giving rise to the melanosomes, responsible for the pigmentation of the skin [6]. Merkel cells on the other hand, are only present in the basal cell layer in small amounts and their main role is related with the

Figure 1.2: Epidermis structure comprised of the stratum basale, stratum spinosum, stratum granulosum, stratum lucidum and stratum corneum [10].

tactile sensation, being closely associated to the terminal nerves. They are especially abundant in highly sensitive areas like lips and fingertips [13].

Above the stratum basale, the stratum spinosum appears as a highly keratinized layer. This is due to the fact that keratinocytes have started their process of differentiation, understood as the keratinization that give rise to terminally differentiated cells that migrate upwards to form the stratum corneum [14]. The third type of cell that populates this stratum are the Langerhans cells, involved in the immune response against invading pathogens [12].

Sitting on top of the stratum spinosum, the stratum granulosum is mainly composed of still differentiating keratinocytes that will become flatter and lose their nuclei before reaching the final layer: the stratum corneum [15]. This final layer is composed of the nuclei-less keratinocytes that have lost all the organelles and of a lipid barrier that has been secreted by the lamellar bodies that hold together the cells [16]. The main role of the stratum corneum is the defensive function by creating a physical barrier from the environment.

The intercellular space in the epidermis contains hyaluronic acid with a non-homogeneous distribution as evidenced through a biotinylated HA-binding protein that revealed higher amount of HA in the upper spinous and granular layers of epidermis with basal keratinocytes expressing HA intracellularly [17, 18].

Dermo-epidermal junction

The dermo-epidermal junction connects the epidermis with the dermis through anchorage of the basal layer of cells to the basement membrane. This tethering is produced through hemidesmosomes, integrin $\alpha 6\beta 4$, collagen IV and XVII and laminin 5 [19]. The majority of the components that conform the dermo-epidermal junction are produced by keratinocytes lying above and in a less extent by the fibroblast present in the dermis. The basement membrane is connected to the dermis through collagen VII fibrils to the collagen types I and III that form the dermal part of the skin. The main function of the dermo-epidermal junction is to give a mechanical support and join together the dermis and epidermis [7].

Dermis

The dermis is the layer of the skin sitting beneath the epidermis and providing it with structural support, nutrition and vascularization. Several structures can be found in this layer like hair follicles, sebaceous glands and sweat glands [16]. The main cell type present in the dermis are the fibroblasts, which are surrounded by the extracellular matrix (ECM) and are responsible for its maintenance and periodic renewal [20]. Other types of cells are also present, being the most common the adipocytes, macrophages and mast cells. The ECM has three main components in the dermis, the collagen fibres, comprising up to 70% of it [21], the elastic fibres and the extrafibrillary matrix or ground substance.

The dermis is divided into two main layers, the papillary dermis and the reticular dermis. Papillary dermis is the thin layer just beneath the epidermis that fills the space found between the ridges formed by the epidermis into the dermis. This layer is mainly composed of thinner collagen I and III fibres loosely organized filling the space. On the other hand, reticular dermis is

mainly comprised of dense collagen I fibres forming a compact structure parallelly aligned with the skin surface [19]. In reticular dermis is were the elastic fibres are present conferring the tissue with the necessary elasticity [6]; while the ground substance fills up the space between fibres and is composed by glycosaminoglycan's (GAG) like hyaluronic acid (HA) and proteoglycans, which help withstand compressive forces.

Hypodermis

The hypodermis is the deepest layer of the skin and it is composed of adipose tissue that confers the skin its thermal insulating property and also serves as an energy storage reservoir. It is placed between the dermis and the muscle and the main cell type present are the adipocytes. Inside this structure, the blood vessels are found creating a microvasculature that provides nutrition and oxygenation to the whole organ [7].

1.2 Skin damage

1.2.1 Causes, prevalence and economic impact

The skin covers all the surface of our body and is constantly subjected to external agents that can cause damage to it. The main causes for skin damage and loss are chronic wounds, trauma and genetic disorders. Chronic wounds, defined as the wounds that remain open for 3 months and are unable to close by themselves, comprise the ulcerations produced as a consequence of other conditions like diabetes mellitus, pressure and venous return. Among the injuries catalogued as trauma and acute wounds, skin burns are the most prevalent cause for skin damage and an estimated 450.000 patients a year visit the emergency room due to this cause in the US alone [22–24]. Genetic disorders like epidermolysis bullosa or ichthyosis vulgaris also represent a challenge in the overcoming of skin diseases [25].

Depending on the extension of the lesion, skin wounds can be classified as superficial, when only the epidermis and papillary dermis are affected; partial thickness, involving both the dermis and epidermis and finally the full-thickness wounds that arise as a consequence of surgical procedures or very deep injuries

[26].

The economic impact of skin damage is certainly huge for the administrations. Chronic wounds, specially, need of large sums of money in order to be treated, making the wound care expenditure for these conditions to reach 8 billion annually only in the US [27]. This number in conjunction with the 5 million patients in the US with diabetic, pressure or leg ulcers plus the progressive ageing of the population is putting pressure to find a clinical and economical solution to this problem.

Regarding burn injuries, the World Health Organization (WHO) estimates that around 180.000 deaths per year occur as a consequence of burns with 95% of them occurring in low and middle-income countries [24]. Hospital visits and treatment of skin burns account for more than 2 billion US dollars in social and economic cost annually, and the market for advanced treatments that comprise advanced dressings has already surpassed the \$3 billion mark in market size [28, 29]. Taking into account the full size of the skin care market, including the advanced and traditional wound care products, this industry is set to reach a market capitalization of around \$12.45 billion dollars by 2022, converting it in one of the biggest healthcare industry businesses [30]. The following figure shows the evolution of the US burn care market revenue from 2014 to 2025 (Figure 1.3).

Figure 1.3: Burn market size forecast [31].

Knowing the causes and the economic burden that the numbers cited represent, there is a growing need to come up with a cost-effective solution for

the treatment of skin wounds regardless of their nature. In order to do so, the kinetics of wound healing need to be fully studied in order to find appropriate solutions, which potentially involve tissue-engineering techniques [32].

1.2.2 Wound healing

Damage to the skin in one of the forms presented before leads to the immediate response by the body that triggers a process known as wound healing in order to fully reinstate the normal function of the skin [19]. Wound healing comprises a series of stages that lead to the repair of the tissue in the case of humans. Other species are known to be able to regenerate damaged tissues, ability lost for adult humans but retained in the case of injuries that occur to the foetus in what has been called as scarless foetal wound healing [33, 34]. Repair is a complex but not perfect process that leads to the formation of fibrotic tissue and a scar, not fully reinstating the original tissue that was damaged. Upon skin injury, a series of coordinated processes involving cellular components, blood, inflammatory pathways, ECM components, proteins etc. start in a sequential manner in order to restore the integrity of the compromised tissue [35]. Wound healing has been classically divided in three main stages: haemostasis and inflammation, cellular proliferation and remodelling [36].

Haemostasis and inflammatory phase

The first response triggered by the body upon skin injury is the vasoconstriction to reduce the loss of blood. Platelet plug formation due to the exposure of blood to damaged tissue will then trigger the formation of a fibrin clot through the coagulation cascade [36, 37]. This platelet plug and fibrin matrix will serve as the provisional scaffold to repair the tissue and will play a role in the beginning of the inflammatory phase by releasing chemotactic agents that recruit the cells and chemical factors involved in the immune response [38]. The infiltration of neutrophils into the wounded area begins the inflammatory stage in which immune cells like macrophages and lymphocytes will arrive to the injury site and clean the wound from debris reducing the risk of potential bacterial infection [33]. The following figure depicts the first stage of wound healing (Figure 1.4).

Figure 1.4: Haemostasis & inflammatory phase of the wound healing process [36].

Cellular proliferation

The second stage in wound healing is the proliferation phase, in which three main processes occur: re-epithelialization, angiogenesis and granulation tissue deposition [39]. Re-epithelialization begins with the migration of fibroblast and keratinocytes into the wound; they come from surrounding areas attracted by the release of cytokines and growth factors (epidermal growth factor (EGF), keratinocyte growth factor (KGF) and transforming growth factor ($\text{TGF-}\beta$)) in the injured area. Simultaneously, new vasculature is formed in a process called angiogenesis in order to provide with oxygen and nutrients to the proliferating cells. The main drivers for angiogenesis are the vascular endothelial growth factor-a (VEGF-A) and fibroblast growth factor-2 (FGF-2) [40]. Finally, granulation tissue deposition occurs as fibroblast start to replace the fibrin matrix with newly deposited collagen III and other ECM components. During this phase, some fibroblasts differentiate into myofibroblast with a contractile capacity to close the wound [33, 41, 42]. Figure 1.5 shows how the cellular proliferation phase occurs.

Remodelling

The final step of the wound healing process starts 2 weeks post injury and mainly involves the stop of all the processes that started with the skin damage. Most of the cells involved in inflammatory response and proliferative phase commit apoptosis, leaving a mainly collagen matrix forming the bulge of the

Figure 1.5: Cellular proliferation phase of the wound healing process [36].

scar [36]. For several months changes in this matrix will keep occurring as collagen III is replaced by stronger collagen I and fibres align with the direction of the wound [43]. The original strength of the tissue is never recovered although a series of new crosslinkings aim to reinforce the scar tissue, reaching a 70% tensile strength with respect to the original unwounded skin [37, 44]. Figure 1.6 illustrates the final aspect of a wound after closure and remodelling.

Figure 1.6: Final aspect of the wound after remodelling phase and closure [36].

1.2.3 Wound management and skin burns treatments

Extensive skin wounds often require the removal of the injured tissue and its replacement with new tissue via surgery procedures. This is mainly the case for skin burns that completely destroy the skin inhibiting the capacity of the tissue to trigger the wound healing and thus compromising the life of the patient [45]. In such cases, several treatments have been used historically, all of which involve the cover of the injured area with healthy tissue capable of regenerating the skin.

The first solution that was used dates back to the end of the ninetieth century and involved the use of a healthy part of skin from the patient in order to expand it and transplant it into the damaged area [46]. This technique is what we call today an autograft and has been widely used in small burn injuries that did not need large areas of healthy skin surface. This type of surgical procedure can use full-thickness skin grafts, dermis and epidermis or split-thickness grafts with just the epidermal and top part of the dermis involved. In the case of extensive burns covering more than 50% of the body surface this technique becomes almost unviable due to the fragile state of the patient and lack of healthy skin sites. The solution to this problem consisted on the expansion of the grafts using different meshing sizes in order to be able to cover the whole injury [19, 47]. Furthermore, repeated extraction of grafts from the same site after quick re-epithelialization (2-3 weeks) can be done but compromising the final scar formation [48].

Due to the persisting issues related with the use of skin from the patient, allografts appeared as an optimal solution. This technique involved the same procedure as the autograft but with the skin either coming from a donor or a cadaveric source. The use of allografts to treat severe burns was first described in 1981 and prevented the wound site from excessive fluid loss and wound desiccation [49]. However, allografts also present drawbacks in terms of tissues compatibility between donor and patient. This has been overcome by removing the epidermal part of the graft after the dermal part has been vascularized and integrated into the patient injury, using then skin grafts from the patient to re-epithelialize the burnt area [5].

These techniques have been in use for several decades with persisting issues in their implementation. That is the main reason why the scientific community needed to find a better solution for this growing problem. In that sense, the

appearance of dermal substitutes that could avoid all the complications related with the use of autografts and allografts has been of vital importance for the development of a complete new field of research. The use of dermal substitutes is still not very wide despite being on the market since 1996 [50], mainly due to its high cost. It is thus imperative to continue working in developing a cost effective solution to this type of skin wounds that both improve the use of surgical grafts and is commercially viable for the administrations. This is where recent developments in the tissue-engineering field need to be applied in order to obtain the definitive solution to chronic and extensive skin injuries.

1.3 Tissue Engineering (TE)

The Tissue Engineering (TE) term was first coined in 1993 and can be defined as the use of a combination of techniques coming from the engineering and life sciences fields with the purpose of the comprehension of the function and structure of tissues both under normal and pathological conditions with the aim to generate substitutes to restore, maintain or improve the tissue function [51].

The initial goal for the TE field from its conception was the development *in vitro* of organs in the laboratory to be ready for transplantation into patients in need. However, after more than 25 years of continuous research the concept and objective has broadened into not only its first main goal but also to the design and testing of new drugs leading to the stage at which organs transplants are not necessary any more [52].

Research has been continuous for these 25 years, but the initial hype and progress envisioned in the first days of the TE field has not translated in an immediate solution for all the existing problems. Development of new tissues in the laboratory has progressed at a much slower pace mainly due to the great complexity of biological tissues [53, 54]. The basis for the correct design of a tissue engineered construct is the combination of cellular components with a physical supporting scaffold that might be active and biodegradable in a controlled manner preferentially and with different chemical and physical signals that guide the overall construct to the regeneration of complex tissues [55]. This environment needs to be perfectly designed to make the cells behave as if they were in an *in vivo* system, including the cellular signalling, diffusion and

disposal of nutrients and wastes respectively and the mechanical and biochemical cues needed for the correct function of tissues [56]. The combination of techniques, cells, scaffolds and cues is resumed in figure 1.7.

Figure 1.7: Techniques involved in the development of new TE therapies [57].

Several examples of TE successes involve mainly the use of natural materials and their application to tissues with relatively low complexity like skin and cartilage. Among those successes, engineered skin for the treatment of burns and chronic ulcers have been reported in several publications [58]. The capability to engineer those tissues lays in their minimal need of vascularisation, which is the main hurdle in the achievement of the TE objectives.

1.3.1 Cell encapsulation in 3D matrices

One of the key aspects for the correct design of an engineered tissue is the use of a three-dimensional scaffold capable of supporting cell encapsulation and growth. The shift from 2D to 3D constructs was a key achievement in order to gain a better insight into the behaviour of cells *in vivo*. Historically, laboratories have used 2D culture conditions in order to replicate processes in the body, research that has lead to most of the medical and pharmaceutical advances that we have today [59]. However, it is increasingly evident that the majority of the basic research produced in the last century has failed to replicate *in vitro* the main aspect of the cellular behaviour in living tissues. This is the reason that has lead the scientific community and specially the professionals of multiple disciplines including tissue engineering to come up with a reliable platform for 3D cell culture that fully resembles the mechanical and biochemical aspects of a living organism [60].

The main difference between 2D and 3D cultures resides in the morphology adopted by the cells (Figure 1.8). Cells in 2D feel a very stiff substrate beneath them that force them to adopt an apical-basal polarity, making them to spread just in the XY plane and altering their phenotype [61]. This type of cell culture might be capable of resembling the behaviour of epithelial cells *in vivo* but it has failed to replicate it for cells in mesenchymal tissues that are normally surrounded by a much softer ECM. Furthermore, the mechanism by which cells interact and adhere to their surrounding has been found to be strikingly different in this two culture systems. While in 2D the primary method for cell adhesion is both through integrins and focal adhesions, in the case of 3D cultures it has been found to be limited to the integrin-based adhesions in the entire cell surface, something that has been found to affect the cells phenotypically [61, 62].

It has become evident in the last decade that the stiffness of the substrate greatly affects the behaviour of the cells by de-regulating signalling pathways leading to changes in the capacity of cells to transform the surrounding matrix in terms of migration, contraction and remodelling [63, 64]. Other aspect that differentiates 2D and 3D cell culture techniques is the presentation of biochemical cues that are heavily present *in vivo* and that modulate the behaviour of cells in the case of injury or matrix disruption [65]. A comprehensive list of factors affecting cell behaviour is depicted in figure 1.9.

Figure 1.8: Different factors affecting cell culture in 2D vs. 3D platforms [60].

1.3.2 Design criteria for the optimal engineered tissue

The process of designing the appropriate scaffold for tissue engineering needs to be carefully studied in order to fulfil several requirements. These requirements will vary from tissue to tissue with the aim of mimicking the natural structure and behaviour of the ECM they intend to replace. The main criteria taken into account can be resumed as follows [66]:

1. The first main characteristic the scaffold must possess is a level of architecture that allows for the formation of the new tissue, that is allow for vascularisation if needed; provide a known rate of degradation in line with the creation of new tissue by the cellular component and being porous enough so that soluble components can diffuse and permit the tissue integration after implantation.

Figure 1.9: Factors affecting cellular behaviour *in vivo* [65].

2. The second most immediate requirement is for the scaffold to be biocompatible and do not elicit an immune response upon implantation into the patient. Furthermore, the scaffold must allow the attachment, growth and differentiation of the cellular component both *in vitro* and *in vivo*.
3. In order to fulfil one of the requirements by definition of TE, scaffolds need to possess some degree of bioactivity. This might include the presentation of cues to the cells, topographical features to guide cellular alignment or the release of active biomolecules in a controlled manner to tune differentiation spatially and temporally.
4. Finally, the scaffolds designed to function as TE solutions must have certain mechanical properties aligned with the tissue they aim to replace temporarily. The scaffolds are intended to maintain and hold the cells and other components structurally for the duration of the regeneration process. Therefore they must completely mimic the native tissue in order to trigger the correct regeneration, including their progressive degradation maintaining their bulk mechanical properties with time. Recently, mechanical properties have been known to affect the phenotype, morphology and adhesion to the matrix of various cell types due to the stiffness

of the substrates they were encapsulated in [67].

1.3.3 Tissue engineered skin substitutes

Parallel to the progresses made in the serial culture of keratinocytes [68] and the development of the TE field, solutions to skin damage appeared first in the form of cultured epithelial autografts (CEAs) for the treatment of extensive burns [69, 70]. Several commercial products using this technique reached the market, like EpiSkinTM [71]; EpicelTM [72] or MySkinTM [73]. More recently, new products that involve the spraying of keratinocytes in the wounded area have appeared in the market, the most representative of them being ReCellTM [74]. Some of the advantages of using this technique are the reduced culture times, higher cell proliferation and generation of a more consistent epithelium [75].

Moving from the epithelial solutions to the dermal substitutes, several products have been commercially available that mimic the dermis to induce the reepithelialisation of full-thickness wounds [76]. Some of these products have just used decellularized collagen matrices from human, porcine or bovine sources [77]. Representative examples are AllodermTM [78]; PermacolTM [79]; IntegraDRTTM [50] or EZDermTM [80]. The absence of a cellular component in these products makes the infiltration of cells and cytokines from the wound bed critical for their correct performance [19]. Other products falling into the dermal category have implemented the incorporation of allogeneic cells into the matrices, like DermagraftTM (Organogenesis, Inc.) [81], better inducing the natural wound healing response of the body although increasing the risk of rejection from the patient [76].

The last type of TE solution for skin damage has brought to the market products that consist on both a dermal and an epidermal layer containing fibroblast and keratinocytes respectively. These matrices are known to enhance the reconstruction of the wounded skin by the formation of the basement membrane, better resembling the natural structure of skin [82]. This improvement comes from the presence of all the components of natural skin but the high cost of these TE products is limiting their implantation mainly due to their low life span [77]. Some of the products that fall into this category are ApligrafTM [83]; TissueTech Autograft SystemTM [84] or PermaDermTM [85].

1.4 Hydrogels in Tissue Engineering

Hydrogels appear as the perfect system to mimic the ECM due to their water-nature and resemblance to the natural tissues. This is one of the reasons why researchers have concentrated on the use of these constructs to design their TE scaffolds in order to regenerate tissues. The definition of hydrogels comes from Peppas *et al.* [86], that defines them as cross-linked polymeric networks that are water-swollen and contain either covalent bonds between one or more monomers; physical interactions between chains; secondary bonds like van der Waals and hydrogen bonds or crystallites bridging two or more macromolecules.

Hydrogels are made of hydrophilic, soluble polymers that form a network through diverse type of crosslinking rendering them insoluble in water and thus trapping great amounts of it in their structure [87]. Depending on their nature hydrogels can be classified as derived from natural sources or synthetic polymers. Further classification is made regarding the mechanism by which the hydrogels are formed (Figure 1.10), being the most common the covalent bonding, physical entanglement and ionic interactions. This crosslinking prevents the soluble entities forming the hydrogel to dissolve in the presence of an aqueous solution [88, 89]. Hydrogels were first reported in the literature by Wichterle and Lim back in the 1960s involving the use of 2-hydroxyethyl methacrylate as soft contact lenses [90]. Since that very first paper the significance of hydrogels in various fields like agriculture [91], pharmaceuticals [92] and the biomedical field [93] has not stop growing. A key aspect in the development of hydrogels for the TE field was the embedding of cells inside them first reported by Lim and Sun in 1980 [94].

The mechanism of formation of hydrogels completely determines their properties in terms of consistency, mechanical properties, degradation behaviour, potential toxicity and biological activity. Chemically formed hydrogels tend to be stronger by the shear nature of their bonds, forming irreversible reactions. Several considerations need to be made when forming hydrogels through the chemical reaction route; first, the reaction must be mild and highly efficient in order not to leave unreactive by-products. Secondly, crosslinkers involved in the reaction must present functional groups and be soluble in the same solvent

Figure 1.10: Mechanisms by which hydrogels can be formed: A) Thermo condensation B) Self assembly C) Ionic gelation D) Electrostatic interaction E) Chemical crosslinking [95].

as the polymeric chains in order for the reaction to be able to occur [96].

The second most used method for hydrogels formation is the physical crosslinking. It involves the use of non-covalent bonds like hydrogen bonds, ionic interactions, hydrophobic forces and molecular self-assembly (Figure 1.10). The mechanical properties of this type of hydrogels can range from very strong and stable gels to soft gels with reversible reactions and thus a limited lifespan [97]. One of the advantages of physically crosslinked hydrogels lies in their versatility and capability to respond to changes in the environment dynamically [96]. However, the difficult tuning of their degradation rates and their poor mechanical properties render them less practical for TE purposes.

Regarding the origin of the materials that form the hydrogels, these can be classified as natural or synthetic. Another type of hydrogels, called hybrid and involving both natural and synthetic materials has appeared as a potential solution for the drawbacks of each of the previously mentioned systems.

1.4.1 Synthetic hydrogels

There exists a trend of thought in TE that argues in favour of the use of synthetic materials that instead of trying to replicate the complexity of tissues *in vivo*, present the cells with the environment to unlock the natural response of the body for self-repair [98]. Furthermore, the use of synthetic materials to make hydrogels present several advantages for the regeneration of tissues, like their scale-up potential for use as commercial regenerative products, their tuneable properties both chemically and physically, their low or non-existent variability batch to batch and their biocompatibility in the majority of the cases [99]. In spite of these advantages, the inert nature of these gels renders them unsuitable for cell adhesion and spread due to their lack of binding sites, only circumvented by the biofunctionalization of the matrices. Furthermore, their synthesis via chemical reactants must be tightly controlled in order to avoid potential toxicity from unreacted species, inhibiting their use for biomedical purposes [99]. The use of this synthetic chemistry also affects the biodegradability of the hydrogels due to the lack of natural cleavable sites that can be detrimental for the correct substitution of the matrix by the regenerated tissue.

Some of the synthetic materials used to generate hydrogels for TE are poly (lactic-co-glycolic) acid (PLGA), poly (ethylene) glycol (PEG), poly (oxazolines) (POx), poly (vinyl alcohol) (PVA) or poly (acrylic) acid (PAA) [100–103].

1.4.2 Natural hydrogels

Hydrogels coming from materials of natural origin were the first used in the literature to try to mimic the skin ECM. Due to their readily availability, biocompatible precursors, great biological properties and biodegradable structures via enzymatic or hydrolytic reactions, natural hydrogels have been used to develop TE skin substitutes that help the regeneration of damaged tissues [99]. Regarding biocompatibility and presentation of molecular and morphological cues to cells to tune their behaviour, natural materials present a clear advantage over the synthetic ones. However, the use of these materials also presents a series of disadvantages with respect to synthetic materials, the most common being the difficulty to chemically modify the natural materials to tune their

properties or their limitations in terms of mechanical properties. Other aspects like the potential variability of the material for different batches and the difficulty to mass-produce them for commercial purposes greatly limit the engineers in their efforts to design the perfect hydrogels for skin TE [104].

Albeit these series of disadvantages, the great features that are naturally associated to natural materials far outweighs the drawbacks just mentioned. The presence of motifs recognisable by cells, the role of these materials in the natural repair of tissues, which is not completely understood and cannot be easily mimicked by synthetic platforms, and their safe degradation products are some of the key aspects that convert natural hydrogels in the perfect matrices for tissue regeneration [104].

The most common natural materials used for the synthesis of skin substitutes have been ECM proteins like collagen, elastin, agarose, alginate, chitosan, fibrin or hyaluronic acid [105, 106].

1.5 Fibrin hydrogels

Fibrin hydrogels are the result of a complex series of reactions naturally occurring upon tissue damage that end with the polymerization of fibrin monomers derived from fibrinogen molecules during the coagulation cascade [107]. The formation of this network is one of the first steps in the wound healing response and is the first scaffold that cells find upon tissue damage [108].

1.5.1 Fibrinogen structure and fibrin polymerization

Fibrinogen is a 45nm in length glycoprotein formed by three pairs of polypeptide chains $(A\alpha B\beta\lambda)_2$ bound together through 29 disulphide bonds and present in the blood stream as a soluble structure with concentrations around 2.5g/L [109]. It consists on two D modules formed by the three polypeptide chains $(A\alpha B\beta\lambda)$ connected through the N-terminal E central module through disulphide bonds [110]. C-terminals of the polypeptides $B\beta$ and λ are present in the distal D modules, while the $A\alpha$ chain folds and binds to the αC -domain connected to the E central module (Figure 1.11)[108].

Figure 1.11: Structure of three fibrinogen monomer and their interaction to form fibrin polymer [107].

Fibrin polymerization starts with the cleavage of the N-terminal fibrinopeptides of A α (FpA) and B β (FpB) chains by activated thrombin. Thrombin is activated through the factor X from prothrombin in the coagulation cascade. Coagulation cascade is the series of enzymatic reactions produced in blood upon tissue damage (extrinsic pathway) or the contact of blood with sub-endothelial tissue mainly collagen (intrinsic pathway) (Figure 1.12). The extrinsic pathway is activated by the contact with thromboplastin or tissue factor (TF) after damage, which in turn activates Factor VII forming a complex with Ca^{2+} that transforms Factor X into Factor Xa, where both pathways converge. The intrinsic pathway involves a more complex series of proteolytic chain reactions that starts with the conversion of Factor XII into Factor XIIa mediated by Kininogen. Factor XIIa then activates Factor XI, whose active form in turn activates Factor IX. This factor then in combination with Factor VIII, phospholipids and Ca^{2+} transforms Factor X into its active form, reaching the common pathway of the coagulation cascade. The common pathway is characterised by the conversion of prothrombin into thrombin mediated by Factor V, Factor Xa and Ca^{2+} , thrombin then catalyses the cleavage of FpA and FpB transforming the fibrinogen monomer into fibrin molecule and starting its polymerization [4].

Polymerization of fibrin continues as the cleaved sites in FpA expose “knobs” A that are complementary to always present “holes” a in the C-terminal of λ -chains. Similarly, although FpA is cleaved more rapidly than FpB, these last fibrinopeptide sites also show “knobs” B upon cleavage that in turn interact with “holes” b in the B β C-terminal of fibrin (Figure 1.13)[107]. It has been demonstrated that the A:a interactions are very stable and strong enough to form the fibrin clot without the need of B:b interactions [112, 113].

Figure 1.12: Schematic of the pathways and factors involved in the coagulation cascade [111].

This interaction between “knobs” and “holes” creates 22.5nm of periodicity two-stranded protofibrils that lengthen with the addition of monomers to each side. These protofibrils can laterally aggregate in a process still unknown that occurs when the length of the protofibrils reach a threshold [114]. Several mechanisms are known to contribute to the lateral aggregation of the protofibrils: the B:b interactions, λ -chains C-terminals and two β -modules of another monomer, the C-terminals of the $\text{A}\alpha$ chain also known as αC region and the coiled coils [115, 116]. The formation of the αC polymer between to αC regions is mediated through the Factor XIIIa (transglutaminase) enhancing the lateral aggregation process but not been determinant to its formation, with thinner fibres formed

when this interactions are hindered [117]. Furthermore, Ca^{2+} plays an important role in the polymerization and subsequent lateral aggregation of fibrin by binding to affinity sites both in the λ -nodules and β -nodules and by increasing the thickness of the fibres as its concentration increases [107]. The final step in the polymerization and formation of a fibrin gel is the branching and formation of a 3D network, which can occur as two pairs of two protofibrils laterally aggregate to later diverge at a point forming a bilateral junction [118] or when the addition of molecules occur via only one λ -nodule making it possible to the elongation of the protofibril in 3 different directions, creating the trimolecular junction (Figure 1.13) [119].

1.5.2 Human plasma-derived bilayered skin equivalents

All the components necessary for the fibrin hydrogel formation are readily present in soluble form in human blood plasma. Blood plasma is separated from the whole blood components through centrifugation and its main composition is 91.5% water and 8.5% solutes, mainly proteins among which fibrinogen is one of the largest and plays a crucial role in the clotting cascade and body haemostasis upon damage [4]. Proteins present in plasma and their concentrations are resumed in table 1.1.

Protein	Molecular weight (kDa)	Concentration (mg/ml)
Albumin	67	40
IgG	150	8-17
LDL	2000	4
HDL	170	3
Fibrinogen	340	2-3
Transferrin	77	2.3
IgA	150	1-4

Table 1.1: Proteins present in human plasma and their molecular weight and concentrations. Adapted from [120].

Fibrin matrices present a series of characteristics that render them a perfect solution for skin damage as TE product. The natural origin of fibrin and its role in the natural wound healing process confers these matrices with great biocompatibility, cell adhesion properties with α -chains motifs like RGD [121],

Figure 1.13: Fibrin polymerization, lateral aggregation and branching mechanism [107].

cell motility during wound healing [122], great cell proliferation and presence of growth factors [123], seamlessly degradation with no by-products via the proteolytic enzyme plasmin and no immune response upon transplantation due to the potential autologous nature [124].

In this thesis, the model followed was the previously reported by Meana *et al.* [125]; Llamas *et al.* [126] and protected by the patent (US2007/0275461A1)[127] held by our group. The group developed a plasma derived fibrin hydrogel for the dermal component with embedded human primary fibroblast and a layer of

human primary keratinocytes seeded on top to form a bioengineered bilayered skin equivalent, also known as organotypic skin construct (Figure 1.14). This skin substitute takes 21 days to fully mature into a final skin *in vitro*; while for transplantation purposes it can be ready in 72 hours. The dermal component needs 24 hours to leach out the excess of calcium chloride needed for hydrogel formation and the human primary keratinocytes seeded on top are left to sink in culture for 48 hours before transplantation.

Figure 1.14: Method of preparation of dermo-epidermal equivalents developed by our group.

This skin equivalent is intended for extensive burn patients, chronic ulcers like diabetic foot ulcers and pressure ulcers and also for the creation of humanized skin animal models as well as potential *in vitro* platforms for drug and cosmetic testing via its integration in skin-on-a-chip solutions. The engineering behind these full-thickness skin equivalent has allowed to solve the limitations presented by the more classical skin damage solutions, the autografts and allografts. The blood plasma can be obtained from a donor thus avoiding the potential damage to the patient and both the fibroblast and keratinocytes are obtained from a skin biopsy from the patient and expanded *in vitro* in order to obtain large surfaces of bioengineered skin in the case of large burns.

The presence of growth factors and cytokines in the fibrin matrix allows for the natural triggering of the skin repair process and the presence of fibroblast induces the keratinocyte proliferation needed to cover the wound. This solution has been proven to be viable for clinical applications in burns, chronic ulcers and necrotizing fasciitis [128].

However, there exist several persisting issues regarding the mechanical properties of the fibrin matrix, which can be quite fragile during handling *in vitro* and have shown to shrink during their transportation to the clinic. These issues are still trying to be solved and in this thesis a method that introduces hyaluronic acid (HA) inside the matrices is postulated as a solution to enhance the mechanical properties, reduce shrinking and induce better cell proliferation and migration due to its natural role in the wound healing process [129].

1.6 Hyaluronic acid (HA)

1.6.1 Discovery, structure, sources, and role in wound healing

Hyaluronic acid (HA) was first isolated from the vitreous body of bovine eyes by Karl Meyer and John Palmer back in 1934 and was first described as a polysaccharide with very high molecular weight [130]. From that moment on, the research on HA has been continuous, finding new places from where HA can be isolated and defining its structure and properties. This research effort has lead the scientific community to fully characterise the HA molecule, the complete structure of the molecule took almost 20 years to be described by the same group that first discovered HA [131]. HA is now defined as a linear polysaccharide composed of the repeating disaccharide unit consisting on β -1,4-D-glucuronic acid and β -1,3-N-acetyl-D-glucosamine (Figure 1.15) [132]. This repeatable disaccharide is 397Da in molecular weight and HA is considered to belong to the glycosaminoglycan's (GAGs) although is the only one that is not sulphated.

GAGs are linear unbranched heteropolysaccharides with varying molecular weight that contain an amino sugar residue and an uronic acid residue forming a repetitive disaccharide. An exception of this is keratan, formed by a

Figure 1.15: Hyaluronic acid molecular structure [132].

pair of glucose-galactose disaccharide (Figure 1.16). GAGs in natural systems present themselves in the form of O-sulfated, N-acetylated, N-deacetylated and N-sulfated saccharides conferring the structures a polyanionic character and thus a negative charge enhancing their interaction with proteins present in plasma, cytokines or amino acids [133–135]. According to the composition of their disaccharide, GAGs can be classified in four main types: (I) hyaluronic acid (HA); (II) heparin (HP)/ heparin sulfate (HS); (III) chondroitin sulfate (CS)/ dermatan sulfate (DS) and (IV) keratan sulfate (KS) [132, 136].

Sulfated GAGs (HP, HS, CS, DS and KS) occur in the body as part of proteoglycans (PGs), which are large macromolecules consisting on a central core protein bound to a membrane or a lipid where GAGs bind covalently [137]. All sulfated GAGs have in common a relatively short peptide chain weighting less than 50 kDa and the fact that their synthesis occurs in the endoplasmic reticulum and Golgi apparatus. The sulphated nature of their chains allows them to have a high affinity to bound other ECM components and thus increase the variety of PGs present in organisms [132].

Contrary to the rest of GAGs, HA presents itself in free-from, unbound to any other structure with a molecular weight range between 1 to 10 million Daltons and with non-sulfated saccharides in its structure [138, 139]. HA is also synthesized apart from the rest of GAGs, instead of in the endoplasmic reticulum it is formed in the plasma membrane by the family of membrane bound HA-synthase [140]. Three different types of trans-membrane proteins HA-synthase exist: HAS1, HAS2 and HAS3, each of them synthesizing different molecular weights HA. While HAS1 make a wide range of HA molecular

Figure 1.16: Principal glycosaminoglycans (GAGs) structure [136].

weight (MW), from 200 kDa up to 2000 kDa; HAS2 synthesizes large MW HA >2000 kDa and HAS3 is responsible for the lower molecular weight form of HA <300 kDa [141]. Regarding HA degradation, it is mediated by hyaluronidases (HYALs) that transform HA into smaller oligosaccharides, the degradation of HA is not limited to HYALs and can be also mediated by reactive oxygen species reason why HA fragments are found during the inflammatory phase of wound healing [142].

HA is an active macromolecule of the connective tissue with a rapid turnover rate that varies from the 1-30 days in joints, 1-2 days in the skin and 2-5 minutes in the bloodstream [143, 144]. The main source of hyaluronan for commercial purposes has been its extraction from the rooster comb and human umbilical cord (animal sources) and its production in large quantities through bacterial

fermentation [145]. The low amounts of HA found in adult animal tissues (Table 1.2) and the growing demand for HA led the scientific community to find ways to mass produce HA through microbiology. HA was discovered first in animals but soon after its discovery, the polysaccharide was also found to be present in bacteria [146]. This finding opened the race to find a way to mass-produce HA in a single strain of *Streptococcus equi*, with more than 20 patents filled between 1985 and 2002 [147]. However, commercial price of HA remains very high despite these research efforts, with highly purified HA at around \$10,000/kg.

Tissue	Content (mg/ml)
Adult rooster comb	Up to 7.5
Eye vitreous body	0.1-0.4
Synovial fluid	1.3-4.0
Hyaline cartilage	1.0
Umbilical cord	4.1
Epidermis	0.1
Dermis	0.2-0.5
Amniotic fluid	20

Table 1.2: Hyaluronic acid content in biological tissues [147].

HA has demonstrated to be an interesting material for tissue engineering due to its high viscoelasticity, compression strength and space filling properties [148]. The highly hydrated nature of HA has been exploited to generate matrices for cosmetic and soft tissue regeneration applications [149]. HA is an innate component of the ECM and thus plays very important roles in biological processes like aging, cellular signaling, skin repair, wound healing and tissue organization [150]. The majority of these processes are mediated by HA due to the presence of cell surface receptors CD44, RHAMM and ICAM-1 that drive cellular proliferation, differentiation and migration [151].

Of important relevance for this work, the role of HA in wound healing has been thoroughly studied [129, 152–154]. Due to its non-allergenic and anti-inflammatory nature combined with the mentioned bioactive properties HA seems to be key in the regeneration of skin after damage. During wound healing the levels of HA in the skin vary, with an initial increase by day three and an overall decrease after seven days. Upon skin damage, HA undergoes a process of

decomposition that result in the broken oligosaccharides from the temporal matrix in combination with fibrin [147, 155]. Parallely and due to this decomposition, newly synthesized HA accumulates at the wound site as the wound healing process progresses, with the degradation products of both HA and fibrin playing a role in new blood vessels formation [153]. The fact that increasing levels of HA are found in foetal scarless wound healing suggest the important role of HA in obtaining a perfectly regenerated skin [156, 157]. Finally, clinical application of HA has shown the acceleration of wound healing in severe burn wounds, which is very promising for the constructs described in this thesis [147].

1.6.2 LifecoreTM Biomedical HA

The first type of HA used for this project was an unmodified, commercially available HA derived from microbial fermentation. This pharmaceutical grade product is sold for research purposes only and complies with all the regulatory agencies like ISO 13485 Certified Quality System, European GMP Certification or CE Mark Certification among others that ensure the purity, quality and reproducibility of experiments when using this product. LifecoreTM Biomedical offers a wide variety of sodium hyaluronates with ranging molecular weights from 5 kDa to 1.8 MDa [158]. For this thesis, three different molecular weights were used, 500 kDa, 700 kDa and 1 MDa. Unmodified HA interacts with fibrin through reversible ionic bonds that might limit the reinforcement of the matrix [105, 159].

1.6.3 Chemically-modified HA

HA in its natural form is capable of interacting with fibrin through complex ionic bonds that generate reversible hydrogels with potential low mechanical properties. In that context, researchers have worked to modify HA in order to crosslink the polysaccharide and form strong irreversible hydrogels. These chemical modifications have resulted in the modulation of the HA properties, from mechanically to biological activity changes [160]. The targets for these modifications are the glucuronic acid carboxyl group, the hydroxyl groups and the N-acetyl group [161]. Depending on the final nature of these chemically modified HA, they can be categorised as “monolithic” or “living” HA derivatives, the first ones being incapable of forming new bonds while the second ones

capable of forming covalent bonds in the presence of cells.

In this thesis we will center our attention into the chemical modification of introducing a thiol group in the carboxyl group of HA (Figure 1.17). This modification will produce an “active” HA derivative that can be later crosslinked with a poly (ethylene glycol) diacrylate (PEGDA) (Figure 1.18) in the presence of cells through the orthogonal chemistry via Michael addition (Figure 1.19). Michael addition has been extensively investigated as the perfect reaction to encapsulate cells into 3D systems and it involves the reaction of a enolate-type nucleophile as the reactive form of a thiol and an unsaturated carbonyl to generate an strog bond [162]. This type of chemistry is characterised by its high efficiency, procedure under mild conditions, no side-products generation, specificity for thiols and insensitivity to water and oxygen presence during the reaction [163, 164].

Figure 1.17: Thiol-modified hyaluronic acid molecular structure [165].

The crosslinker used to generate HA hydrogels was a derivate of the widely known poly (ethylene) glycol (PEG) polyester. Some of its properties are great biocompatibility and hydrophilicity making it the perfect polymer for TE purposes [166]. PEG can appear as a linear or branched structure and does not naturally contain cell-adhesion motifs, thus biofunctionalization when used isolated is needed for cellular applications [167]. PEG contains two hydroxyl groups at its ends that can be chemically modified to contain other functional groups like amines, thiols, azide, vinyl sulfone or acrylates [168]. For our crosslinking pur-

poses a PEG containing acrylates groups at its ends was selected to perform the crosslinking of thiol-modified HA.

Figure 1.18: Poly-ethylene glycol diacrylate (PEGDA) [165].

Figure 1.19: Michael addition reaction procedure. Adapted from: [169].

Properties, like mechanical strenght, degradation rate and biological activity of the generated hydrogels can be modulated depending on the parameters of the crosslinking. In this way, initial molecular weight of HA, percentage of thiol-modified groups, molecular weight of the crosslinker (PEGDA) and the ratio between thiols and acrylates are key factors for the final HA hydrogel product [170].

1.6.4 ESI-Bio HyStem® hydrogels

In this thesis, a commercially available, animal-free hydrogel system consisting on a thiol-modified HA (Glycosil®) with 240 kDa MW and a polyethylene diacrylate (PEGDA) (Extralink®) crosslinker with 3400Da MW were used. This system is designed to encapsulate cells in a 3D matrix fully controlling the gelation time and mechanical properties of the hydrogels. These matrices

Figure 1.20: Thiol-modified HA crosslinked with PEGDA [165].

are chemically defined and form a synthetic matrix that complies with Good Manufacturing Practices (GMP) and the European Pharmacopoeia production protocols [171].

The HA is produced through a bacterial fermentation in *Bacillus subtilis* under a regulated ISO 9001:2000 process free of organic solvents and animal-derived materials. In terms of chemical modifications, HA contains 1 μmol of thiol for every mg of Glycosil[®] while PEGDA contains 0.58 μmol of acrylate groups for every mg of Extralink[®] [171]. These numbers allowed us to design several crosslinking ratios relating the number of thiol moles to acrylates moles (2:1, 6:1, 10:1 and 14:1) in order to prepare hydrogels with varying stiffness.

These two products form part of what BioTime calls the HyStem[®] hydrogels systems which have already been used in clinical trials with the name of Renevia[®]. The trial aimed to overcome the HIV-associated lipodystrophy in the face of patients. Successful results in this trial have led the company to apply for the CE certification in Europe due to the good volume retention results of 100% of the product after 6 months [172].

Figure 1.21: HyStem[®] hydrogels kit from [171].

1.7 Motivation

The rationale behind this thesis lies in the necessity to improve the current solutions being applied to patients for severe skin damage and to generate a reliable *in vitro* platform for toxicity, cosmetical and pharmaceutical research [173]. Nowadays, autografts and allografts have been the main driver in the reconstruction of skin defects as results of acute damage, extensive burns and chronic ulcers. The use of fibrin-based matrices is of key importance as the results obtained have outperformed the ones obtained for autografts and allografts. This smooth solution has allowed the development of dermo-epidermal equivalents with cells of autologous origin for the treatment of the aforementioned conditions.

However, the use of these full-skin TE constructs has not been widely spread due to the high cost and several drawbacks such as the time needed for their preparation. In the case of the fibrin matrix patented system of our group, it has been extensively used and persisting issues have been reported that are hindering the application of this system in its full potential. These issues comprise the high degradation rates found for fibrin hydrogels [174] and their poor mechanical properties compromising the handling and stability of the scaffolds

before (*in vitro*) and after implantation (*in vivo*) [175]. In this context, new ways of improving the matrices have arisen in the form of incorporation of new materials that can confer new properties to the scaffolds. In this thesis we present a thoroughly and profound study of the reasons behind the persisting issues reported for plasma-derived matrices and propose a solution in the form of the incorporation of hyaluronic acid (HA) at physiological concentrations (0.05% - 0.2%) in order to overcome them. HA is a glycosaminoglycan (GAG) naturally present in the skin and with an important role in the wound healing process. In that sense it is presented as the optimal solution for both improving the properties of the scaffolds in terms of mechanics and contraction and also better mimicking the natural environment in which cells encounter themselves to repair the skin damage.

1.8 Thesis outline

The chapters that follow this introduction to the topic of skin TE will be devoted to the presentation of the experiments performed and results obtained in order to achieve the objectives of this work. First, plasma hydrogels will be studied in terms of final fibrin concentration present in the formulation to seek for differences that can be accountable for the persisting issues encountered. Secondly, the effect of the external environment affecting the behaviour of plasma-derived matrices will be studied with experiments regarding the effect of temperature in the matrix. Thirdly, incorporation of two types of HA into the structure of the matrices will be studied with the objective to improve the matrices in terms of inhibiting the contraction, the degradation and improving the mechanical properties. Furthermore, these matrices with incorporated HA will be used to generate organotypic skin constructs *in vitro* and its performance will be eventually evaluated *in vivo* by transplanting them into mice. Finally, conclusions for this thesis will be presented.

Bibliography

- [1] M. J. Escámez, L. Martínez-Santamaría, M. García, S. Guerrero-Aspizua, M. Carretero, F. Larcher, A. Meana, and M. Del Río, “Bioengineered skin,” in *Skin Biopsy-Perspectives*, IntechOpen, 2011.
- [2] T. P. Habif, *Clinical Dermatology E-Book: A Color Guide to Diagnosis and Therapy*. Elsevier Health Sciences, 2015.
- [3] A. Charruyer and R. Ghadially, “Stem cells and tissue-engineered skin,” *Skin pharmacology and physiology*, vol. 22, no. 2, pp. 55–62, 2009.
- [4] G. J. Tortora and B. Derrickson, *Principles of anatomy & physiology*. John Wiley & Sons, Incorporated, 2017.
- [5] S. MacNeil, “Progress and opportunities for tissue-engineered skin.,” *Nature*, vol. 445, pp. 874–880, feb 2007.
- [6] W. K. Ovalle and P. C. Nahirney, *Netter’s Essential Histology: with Student Consult Access*. Elsevier Health Sciences, 2013.
- [7] W. D. James, D. Elston, and T. Berger, *Andrew’s Diseases of the Skin E-Book: Clinical Dermatology*. Elsevier Health Sciences, 2011.
- [8] S. Paxton, M. Peckham, and A. Knibbs, “The Leeds Histology Guide,” 2003.
- [9] V. Kumar, A. K. Abbas, N. Fausto, and J. C. Aster, *Robbins and Cotran pathologic basis of disease, professional edition e-book*. Elsevier health sciences, 2014.
- [10] OpenStax, “5.1 Layers of the Skin | Anatomy and Physiology. Retrieved from: <https://openstax.org/books/anatomy-and-physiology/pages/5-1-layers-of-the-skin>,” 2013.
- [11] W. L. Weston, A. T. Lane, and J. G. Morelli, *Color Textbook of Pediatric Dermatology E-Book*. Elsevier Health Sciences, 2007.
- [12] D. J. Wong and H. Y. Chang, “Skin tissue engineering,” in *StemBook [Internet]*, Harvard Stem Cell Institute, 2009.
- [13] S.-H. Woo, E. A. Lumpkin, and A. Patapoutian, “Merkel cells and neurons keep in touch,” *Trends in cell biology*, vol. 25, no. 2, pp. 74–81, 2015.

- [14] S. Shetty and S. Gokul, “Keratinization and its disorders,” *Oman medical journal*, vol. 27, no. 5, p. 348, 2012.
- [15] J. E. Fitzpatrick and J. G. Morelli, *Dermatology Secrets Plus E-Book*. Elsevier Health Sciences, 2010.
- [16] J. G. Marks and J. J. Miller, *Lookingbill and Marks’ Principles of Dermatology E-Book*. Elsevier Health Sciences, 2017.
- [17] R. Tammi, J. A. Ripellino, R. U. Margolis, and M. Tammi, “Localization of epidermal hyaluronic acid using the hyaluronate binding region of cartilage proteoglycan as a specific probe,” *Journal of Investigative Dermatology*, vol. 90, no. 3, 1988.
- [18] U. Berthelm, S. Hellstro, *et al.*, “The distribution of hyaluronan in human skin and mature, hypertrophic and keloid scars,” *British journal of plastic surgery*, vol. 47, no. 7, pp. 483–489, 1994.
- [19] M. Albanna and J. H. Holmes IV, *Skin tissue engineering and regenerative medicine*. Academic Press, 2016.
- [20] M. Mahdavi-Mazdeh, B. N. Heshmati, S. A. H. Tavakoli, M. Ayaz, F. A. Ardalan, and M. Momeni, “Human split-thickness skin allograft: skin substitute in the treatment of burn,” *International journal of organ transplantation medicine*, vol. 4, no. 3, p. 96, 2013.
- [21] D. Gawkrödger and M. R. Ardern-Jones, *Dermatology E-Book: An Illustrated Colour Text*. Elsevier Health Sciences, 2016.
- [22] S. F. Miller, P. Bessey, C. W. Lentz, J. C. Jeng, M. Schurr, S. Browning, and Committee, “National burn repository 2007 report: a synopsis of the 2007 call for data,” *Journal of burn care & research*, vol. 29, no. 6, pp. 862–870, 2008.
- [23] M. D. Peck, “Epidemiology of burns throughout the world. Part I: Distribution and risk factors,” *Burns*, vol. 37, no. 7, pp. 1087–1100, 2011.
- [24] W. H. Organization, “Burns,” 2018.
- [25] G. M. DeStefano and A. M. Christiano, “The genetics of human skin disease,” *Cold Spring Harbor perspectives in medicine*, vol. 4, oct 2014.
- [26] N. J. Percival, “Classification of Wounds and their Management,” *Surgery (Oxford)*, vol. 20, pp. 114–117, may 2003.

- [27] D. M. Supp and S. T. Boyce, “Engineered skin substitutes: practices and potentials.,” *Clinics in dermatology*, vol. 23, no. 4, pp. 403–412, 2005.
- [28] F. Grinnell and C. R. Lamke, “Reorganization of hydrated collagen lattices by human skin fibroblasts,” *Journal of cell science*, vol. 66, no. 1, pp. 51–63, 1984.
- [29] H. Yanai, A. Budovsky, R. Tacutu, and V. E. Fraifeld, “Is rate of skin wound healing associated with aging or longevity phenotype?,” *Biogerontology*, vol. 12, no. 6, pp. 591–597, 2011.
- [30] M. Adnan Malik, “Advanced Wound Care Market Size, Share and Industry Forecast | 2022,” tech. rep., Allied Market Research, 2016.
- [31] Grand View Research, “Global Burn Care Market Size, Share | Industry Trend Report, 2019-2026,” tech. rep., 2019.
- [32] R. A. F. Clark, K. Ghosh, and M. G. Tonnesen, “Tissue engineering for cutaneous wounds,” *Journal of Investigative Dermatology*, vol. 127, no. 5, pp. 1018–1029, 2007.
- [33] J. M. Reinke and H. Sorg, “Wound repair and regeneration,” *European surgical research*, vol. 49, no. 1, pp. 35–43, 2012.
- [34] A. S. Colwell, M. T. Longaker, and H. P. Lorenz, “Fetal wound healing.,” *Frontiers in bioscience : a journal and virtual library*, vol. 8, pp. s1240–8, sep 2003.
- [35] R. V. Shevchenko, S. L. James, and S. E. James, “A review of tissue-engineered skin bioconstructs available for skin reconstruction,” *Journal of the royal Society Interface*, vol. 7, no. 43, pp. 229–258, 2009.
- [36] G. C. Gurtner, S. Werner, Y. Barrandon, and M. T. Longaker, “Wound repair and regeneration,” *Nature*, vol. 453, no. 7193, p. 314, 2008.
- [37] R. Lanza, R. Langer, and J. P. Vacanti, *Principles of tissue engineering*. Academic press, 2011.
- [38] M. C. Robson, D. L. Steed, and M. G. Franz, “Wound healing: biologic features and approaches to maximize healing trajectories,” *Current problems in surgery*, vol. 2, no. 38, pp. 72–140, 2001.
- [39] L. M. McManus and R. N. Mitchell, *Pathobiology of human disease: a dynamic encyclopedia of disease mechanisms*. Elsevier, 2014.

- [40] S. Werner and R. Grose, “Regulation of wound healing by growth factors and cytokines,” *Physiological reviews*, vol. 83, no. 3, pp. 835–870, 2003.
- [41] S. R. Opalenik and J. M. Davidson, “Fibroblast differentiation of bone marrow-derived cells during wound repair,” *The FASEB Journal*, vol. 19, no. 11, pp. 1561–1563, 2005.
- [42] B. Hinz, “Formation and function of the myofibroblast during tissue repair,” *Journal of Investigative Dermatology*, vol. 127, no. 3, pp. 526–537, 2007.
- [43] H. N. Lovvorn Iii, D. T. Cheung, M. E. Nimni, N. Perelman, J. M. Estes, and N. S. Adzick, “Relative distribution and crosslinking of collagen distinguish fetal from adult sheep wound repair,” *Journal of pediatric surgery*, vol. 34, no. 1, pp. 218–223, 1999.
- [44] G. C. Gurtner and G. R. Evans, “Advances in head and neck reconstruction,” *Plastic and reconstructive surgery*, vol. 106, no. 3, pp. 672–682, 2000.
- [45] Z. Ruszczak, “Effect of collagen matrices on dermal wound healing,” *Advanced drug delivery reviews*, vol. 55, no. 12, pp. 1595–1611, 2003.
- [46] J.-L. Reverdin, *De la greffe epidermique*. P. Asselin, successeur de Béchet jeune et Labé, 1872.
- [47] E. S. Garfein, D. P. Orgill, and J. J. Pribaz, “Clinical applications of tissue engineered constructs,” *Clinics in plastic surgery*, vol. 30, no. 4, pp. 485–498, 2003.
- [48] S. T. Boyce, R. J. Kagan, K. P. Yakuboff, N. A. Meyer, M. T. Rieman, D. G. Greenhalgh, and G. D. Warden, “Cultured skin substitutes reduce donor skin harvesting for closure of excised, full-thickness burns,” *Annals of surgery*, vol. 235, no. 2, p. 269, 2002.
- [49] J. W. Alexander, B. G. MacMillan, E. Law, and D. S. Kittur, “Treatment of severe burns with widely meshed skin autograft and meshed skin allograft overlay,” *The Journal of trauma*, vol. 21, no. 6, pp. 433–438, 1981.
- [50] “Integra® Dermal Regeneration Template « Integra Life-Sciences: Europe, Middle East and Africa. Retrieved from

<https://www.integralife.eu/products/plastic/integra-dermal-regeneration-template-2/>. Access date: 2019-06-03.”

- [51] R. Langer and J. P. Vacanti, “Tissue engineering,” *Science*, vol. 260, pp. 920 LP – 926, may 1993.
- [52] L. G. Griffith and G. Naughton, “Tissue engineering—current challenges and expanding opportunities,” *Science*, vol. 295, no. 5557, pp. 1009–1014, 2002.
- [53] M. J. Lysaght and A. L. Hazlehurst, “Tissue engineering: the end of the beginning,” *Tissue engineering*, vol. 10, no. 1-2, pp. 309–320, 2004.
- [54] M. J. Lysaght, A. Jaklenec, and E. Deweerdt, “Great expectations: private sector activity in tissue engineering, regenerative medicine, and stem cell therapeutics,” *Tissue Engineering Part A*, vol. 14, no. 2, pp. 305–315, 2008.
- [55] L. G. Griffith, “Polymeric biomaterials,” *Acta materialia*, vol. 48, no. 1, pp. 263–277, 2000.
- [56] F. Berthiaume, T. J. Maguire, and M. L. Yarmush, “Tissue engineering and regenerative medicine: history, progress, and challenges,” *Annual review of chemical and biomolecular engineering*, vol. 2, pp. 403–430, 2011.
- [57] A. Khademhosseini and R. Langer, “A decade of progress in tissue engineering,” *Nature protocols*, vol. 11, no. 10, p. 1775, 2016.
- [58] A. Khademhosseini, J. P. Vacanti, and R. Langer, “Progress in tissue engineering,” *Scientific American*, vol. 300, no. 5, pp. 64–71, 2009.
- [59] K. Duval, H. Grover, L.-H. Han, Y. Mou, A. F. Pegoraro, J. Fredberg, and Z. Chen, “Modeling physiological events in 2D vs. 3D cell culture,” *Physiology*, vol. 32, no. 4, pp. 266–277, 2017.
- [60] B. M. Baker and C. S. Chen, “Deconstructing the third dimension—how 3D culture microenvironments alter cellular cues,” *J Cell Sci*, vol. 125, no. 13, pp. 3015–3024, 2012.
- [61] M. A. Schwartz and M. H. Ginsberg, “Networks and crosstalk: integrin signalling spreads,” *Nature cell biology*, vol. 4, no. 4, p. E65, 2002.

- [62] C. A. Reinhart-King, M. Dembo, and D. A. Hammer, "The dynamics and mechanics of endothelial cell spreading," *Biophysical journal*, vol. 89, no. 1, pp. 676–689, 2005.
- [63] F. Grinnell, C.-H. Ho, Y.-C. Lin, and G. Skuta, "Differences in the regulation of fibroblast contraction of floating versus stressed collagen matrices," *Journal of Biological Chemistry*, vol. 274, no. 2, pp. 918–923, 1999.
- [64] M. Ehrbar, A. Sala, P. Lienemann, A. Ranga, K. Mosiewicz, A. Bittermann, S. C. Rizzi, F. E. Weber, and M. P. Lutolf, "Elucidating the role of matrix stiffness in 3D cell migration and remodeling," *Biophysical journal*, vol. 100, no. 2, pp. 284–293, 2011.
- [65] L. G. Griffith and M. A. Swartz, "Capturing complex 3D tissue physiology in vitro," *Nature Reviews Molecular Cell Biology*, vol. 7, no. 3, p. 211, 2006.
- [66] B. P. Chan and K. W. Leong, "Scaffolding in tissue engineering: general approaches and tissue-specific considerations," *European spine journal*, vol. 17, no. 4, pp. 467–479, 2008.
- [67] D. E. Discher, P. Janmey, and Y.-l. Wang, "Tissue cells feel and respond to the stiffness of their substrate," *Science*, vol. 310, no. 5751, pp. 1139–1143, 2005.
- [68] J. G. Rheinwald and H. Green, "Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells," *Cell*, vol. 6, no. 3, pp. 331–343, 1975.
- [69] N. O'Connor, J. Mulliken, S. Banks-Schlegel, O. Kehinde, and H. Green, "Grafting of burns with cultured epithelium prepared from autologous epidermal cells," *The Lancet*, vol. 317, no. 8211, pp. 75–78, 1981.
- [70] C. Cuono, R. Langdon, and J. McGuire, "Use of cultured epidermal autografts and dermal allografts as skin replacement after burn injury," *The Lancet*, vol. 327, no. 8490, pp. 1123–1124, 1986.
- [71] "Episkin - World leader in tissue engineering for in vitro human skin and epithelial models. Retrieved from <http://www.episkin.com/>. Access date:2019-06-03."

- [72] “Epicel. Retrieved from <https://www.epicel.com/>. Access date: 2019-06-03.”
- [73] “Home - Regenerys. Retrieved from <https://regenerys.com/>. Access date:2019-06-03.”
- [74] “AVITA Medical | RECELL | Regenerative Medicine for Skin Conditions. Retrieved from: <https://avitamedical.com/about-recell>. Access date: 2019-06-03.”
- [75] T. Fang, W. C. Lineaweaver, F. C. Sailes, C. Kisner, and F. Zhang, “Clinical application of cultured epithelial autografts on acellular dermal matrices in the treatment of extended burn injuries,” *Annals of plastic surgery*, vol. 73, no. 5, pp. 509–515, 2014.
- [76] E. Catalano, A. Cochis, E. Varoni, L. Rimondini, and B. Azzimonti, “Tissue-engineered skin substitutes: an overview,” *Journal of Artificial Organs*, vol. 16, no. 4, pp. 397–403, 2013.
- [77] R. A. Kamel, J. F. Ong, E. Eriksson, J. P. E. Junker, and E. J. Cater-son, “Tissue engineering of skin,” *Journal of the American College of Surgeons*, vol. 217, no. 3, pp. 533–555, 2013.
- [78] D. Wainwright, “Use of an acellular allograft dermal matrix (alloderm) in the management of full-thickness burns,” *Burns*, vol. 21, no. 4, pp. 243–248, 1995.
- [79] C. Harper, “PermacolTM: clinical experience with a new biomaterial,” *Hospital Medicine*, vol. 62, no. 2, pp. 90–95, 2001.
- [80] “EZDERM | Integrated Dermatology EHR and PM Suite | EZDERM. Retrieved from <https://ezderm.com/>. Access date: 2019-06-03.”
- [81] “About Dermagraft. Retrieved from <http://www.dermagraft.com/patient/about-dermagraft/>. Access date: 2019-06-03.”
- [82] D. Breitkreutz, N. Mirancea, and R. Nischt, “Basement membranes in skin: unique matrix structures with diverse functions?,” *Histochemistry and cell biology*, vol. 132, no. 1, pp. 1–10, 2009.
- [83] “Apligraf : What is Apligraf?. Retrieved from <http://www.apligraf.com/professional/>. Access date: 2019-06-03.”

- [84] L. Uccioli, “A clinical investigation on the characteristics and outcomes of treating chronic lower extremity wounds using the tissuetech autograft system,” *The international journal of lower extremity wounds*, vol. 2, no. 3, pp. 140–151, 2003.
- [85] “Welcome to Regenicin specializing development regenerative cell therapies, NovaDerm®. Retrieved from <https://www.regenicin.com/>. Access date: 2019-06-03.”
- [86] N. A. Peppas, P. Bures, W. S. Leobandung, and H. Ichikawa, “Hydrogels in pharmaceutical formulations,” *European journal of pharmaceuticals and biopharmaceutics*, vol. 50, no. 1, pp. 27–46, 2000.
- [87] A. M. Jonker, D. W. Löwik, and J. C. Van Hest, “Peptide- and protein-based hydrogels,” mar 2012.
- [88] E. M. Ahmed, “Hydrogel: Preparation, characterization, and applications: A review,” *Journal of advanced research*, vol. 6, no. 2, pp. 105–121, 2015.
- [89] P. Dubin, Y. Osada, and H. B. Bohidar, *Polymer gels: fundamentals and applications*. American Chemical Society, 2003.
- [90] O. Wichterle and D. Lim, “Hydrophilic gels for biological use,” *Nature*, vol. 185, no. 4706, p. 117, 1960.
- [91] X. Chen, B. D. Martin, T. K. Neubauer, R. J. Linhardt, J. S. Dordick, and D. G. Rethwisch, “Enzymatic and chemoenzymatic approaches to synthesis of sugar-based polymer and hydrogels,” *Carbohydrate polymers*, vol. 28, no. 1, pp. 15–21, 1995.
- [92] H. Y. Zhou, X. G. Chen, M. Kong, C. S. Liu, D. S. Cha, and J. F. Kennedy, “Effect of molecular weight and degree of chitosan deacetylation on the preparation and characteristics of chitosan thermosensitive hydrogel as a delivery system,” *Carbohydrate polymers*, vol. 73, no. 2, pp. 265–273, 2008.
- [93] A. S. Hoffman, “Hydrogels for biomedical applications,” *Advanced Drug Delivery Reviews*, vol. 64, pp. 18–23, dec 2012.
- [94] F. Lim and A. M. Sun, “Microencapsulated islets as bioartificial endocrine pancreas,” *Science*, vol. 210, no. 4472, pp. 908–910, 1980.

- [95] Y. S. Zhang and A. Khademhosseini, “Advances in engineering hydrogels,” *Science*, vol. 356, no. 6337, p. eaaf3627, 2017.
- [96] S. E. Grieshaber, A. K. Jha, A. J. E. Farran, and X. Jia, “Hydrogels in tissue engineering,” in *Biomaterials for Tissue Engineering Applications*, pp. 9–46, Springer, 2011.
- [97] S. K. H. Gulrez, S. Al-Assaf, and G. O. Phillips, “Hydrogels: methods of preparation, characterisation and applications,” *Progress in molecular and environmental bioengineering—from analysis and modeling to technology applications*, pp. 117–150, 2011.
- [98] E. S. Place, N. D. Evans, and M. M. Stevens, “Complexity in biomaterials for tissue engineering,” *Nature materials*, vol. 8, no. 6, p. 457, 2009.
- [99] B. V. Slaughter, S. S. Khurshid, O. Z. Fisher, A. Khademhosseini, and N. A. Peppas, “Hydrogels in regenerative medicine,” *Advanced materials*, vol. 21, no. 32-33, pp. 3307–3329, 2009.
- [100] I. M. El-Sherbiny and M. H. Yacoub, “Hydrogel scaffolds for tissue engineering: Progress and challenges,” *Global Cardiology Science and Practice*, p. 38, 2013.
- [101] A. J. R. Lasprilla, G. A. R. Martinez, B. H. Lunelli, A. L. Jardini, and R. Maciel Filho, “Poly-lactic acid synthesis for application in biomedical devices—A review,” *Biotechnology advances*, vol. 30, no. 1, pp. 321–328, 2012.
- [102] D. S. Kohane and R. Langer, “Polymeric biomaterials in tissue engineering,” *Pediatric research*, vol. 63, no. 5, p. 487, 2008.
- [103] B. L. Farrugia, K. Kempe, U. S. Schubert, R. Hoogenboom, and T. R. Dargaville, “Poly (2-oxazoline) hydrogels for controlled fibroblast attachment,” *Biomacromolecules*, vol. 14, no. 8, pp. 2724–2732, 2013.
- [104] E. L. Monzack, K. J. Rodriguez, C. M. McCoy, X. Gu, and K. S. Masters, “Natural materials in tissue engineering applications,” in *Biomaterials for Tissue Engineering Applications*, pp. 209–241, Springer, 2011.
- [105] K. Y. Lee and D. J. Mooney, “Hydrogels for tissue engineering,” *Chemical reviews*, vol. 101, no. 7, pp. 1869–1880, 2001.

- [106] M. Klimov, T. Leavitt, J. Molnar, and D. Orgill, “Natural biomaterials for skin tissue engineering,” *Albanna MZ, Holmes IV JH. Skin Tissue Engineering and Regenerative Medicine. Academic Press, Cambridge MA, USA*, pp. 145–161, 2016.
- [107] J. W. Weisel and R. I. Litvinov, “Mechanisms of fibrin polymerization and clinical implications,” *Blood*, vol. 121, no. 10, pp. 1712–1719, 2013.
- [108] P. A. Janmey, J. P. Winer, and J. W. Weisel, “Fibrin gels and their clinical and bioengineering applications,” *Journal of the Royal Society Interface*, vol. 6, no. 30, pp. 1–10, 2008.
- [109] J. W. Weisel, “Fibrinogen and fibrin,” in *Advances in protein chemistry*, vol. 70, pp. 247–299, Elsevier, 2005.
- [110] M. W. Mosesson, “Fibrinogen and fibrin structure and functions,” *Journal of Thrombosis and Haemostasis*, vol. 3, no. 8, pp. 1894–1904, 2005.
- [111] M. A. Petersen, J. K. Ryu, and K. Akassoglou, “Fibrinogen in neurological diseases: mechanisms, imaging and therapeutics,” *Nature Reviews Neuroscience*, vol. 19, no. 5, p. 283, 2018.
- [112] R. I. Litvinov, O. V. Gorkun, S. F. Owen, H. Shuman, and J. W. Weisel, “Polymerization of fibrin: specificity, strength, and stability of knob-hole interactions studied at the single-molecule level,” *Blood*, vol. 106, no. 9, pp. 2944–2951, 2005.
- [113] R. I. Litvinov, O. V. Gorkun, D. K. Galanakis, S. Yakovlev, L. Medved, H. Shuman, and J. W. Weisel, “Polymerization of fibrin: direct observation and quantification of individual B: b knob-hole interactions,” *Blood*, vol. 109, no. 1, pp. 130–138, 2007.
- [114] I. N. Chernysh, C. Nagaswami, and J. W. Weisel, “Visualization and identification of the structures formed during early stages of fibrin polymerization,” *Blood*, vol. 117, no. 17, pp. 4609–4614, 2011.
- [115] Z. Yang, I. Mochalkin, and R. F. Doolittle, “A model of fibrin formation based on crystal structures of fibrinogen and fibrin fragments complexed with synthetic peptides,” *Proceedings of the National Academy of Sciences*, vol. 97, no. 26, pp. 14156–14161, 2000.

- [116] N. Okumura, F. Terasawa, M. Hirota-Kawadobora, K. Yamauchi, K. Nakanishi, S. Shiga, S. Ichiyama, M. Saito, M. Kawai, and T. Nakahata, "A novel variant fibrinogen, deletion of B β 111Ser in coiled-coil region, affecting fibrin lateral aggregation," *Clinica chimica acta*, vol. 365, no. 1-2, pp. 160–167, 2006.
- [117] J.-P. Collet, J. L. Moen, Y. I. Veklich, O. V. Gorkun, S. T. Lord, G. Montalescot, and J. W. Weisel, "The α C domains of fibrinogen affect the structure of the fibrin clot, its physical properties, and its susceptibility to fibrinolysis," *Blood*, vol. 106, no. 12, pp. 3824–3830, 2005.
- [118] M. W. Mosesson, J. P. DiOrio, K. R. Siebenlist, J. S. Wall, and J. F. Hainfeld, "Evidence for a second type of fibril branch point in fibrin polymer networks, the trimolecular junction," *Blood*, vol. 82, no. 5, pp. 1517–1521, 1993.
- [119] A. L. Fogelson and J. P. Keener, "Toward an understanding of fibrin branching structure," *Physical Review E*, vol. 81, no. 5, p. 51922, 2010.
- [120] B. Buis, G. C. Koomen, A. L. Imholz, D. G. Struijk, R. E. Reddingius, L. Arisz, and R. T. Krediet, "Effect of electric charge on the transperitoneal transport of plasma proteins during CAPD.," *Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association*, vol. 11, pp. 1113–1120, jun 1996.
- [121] K. Suehiro, J. Mizuguchi, K. Nishiyama, S. Iwanaga, D. H. Farrell, and S. Ohtaki, "Fibrinogen binds to integrin $\alpha 5\beta 1$ via the carboxyl-terminal RGD site of the A α -chain," *The Journal of Biochemistry*, vol. 128, no. 4, pp. 705–710, 2000.
- [122] N. I. A. Mahboob Morshed, S. R. Chowdhury, and B. H. I. Ruszymah, "The current available biomaterials being used for skin tissue engineering," *Regen Res*, vol. 3, pp. 17–22, 2014.
- [123] B. H. Ruszymah, "Autologous human fibrin as the biomaterial for tissue engineering.," *The Medical journal of Malaysia*, vol. 59, pp. 30–31, 2004.
- [124] C. Buchta, H. C. Hedrich, M. Macher, P. Höcker, and H. Redl, "Biochemical characterization of autologous fibrin sealants produced by CryoSeal® and Vivostat® in comparison to the homologous fibrin sealant product Tissucol/Tisseel®," *Biomaterials*, vol. 26, no. 31, pp. 6233–6241, 2005.

- [125] A. Meana, J. Iglesias, M. Del Rio, F. Larcher, B. Madrigal, M. Fresno, C. Martin, F. San Roman, and F. Tevar, "Large surface of cultured human epithelium obtained on a dermal matrix based on live fibroblast-containing fibrin gels," *Burns*, vol. 24, no. 7, pp. 621–630, 1998.
- [126] S. G. Llames, M. Del Rio, F. Larcher, E. García, M. García, M. J. Escamez, J. L. Jorcano, P. Holguín, and A. Meana, "Human plasma as a dermal scaffold for the generation of a completely autologous bioengineered skin," *Transplantation*, vol. 77, no. 3, pp. 350–355, 2004.
- [127] J. L. Jorcano, F. Laguzzi, A. Infiesta, S. Llanes, and M. Nechaevsky, "Artificial dermis and method of preparation," Nov 2007.
- [128] L. Martínez-Santamaría, S. Guerrero-Aspizua, and M. Del Río, "Skin bioengineering: preclinical and clinical applications," *Actas Dermosifiliográficas (English Edition)*, vol. 103, no. 1, pp. 5–11, 2012.
- [129] W. Y. J. Chen and G. Abatangelo, "Functions of hyaluronan in wound repair," *Wound repair and regeneration*, vol. 7, no. 2, pp. 79–89, 1999.
- [130] K. Meyer and J. W. Palmer, "The polysaccharide of the vitreous humor," *Journal of Biological Chemistry*, vol. 107, no. 3, pp. 629–634, 1934.
- [131] A. Linker and K. Meyer, "Production of unsaturated uronides by bacterial hyaluronidases," *Nature*, vol. 174, no. 4443, p. 1192, 1954.
- [132] M. Mende, C. Bednarek, M. Wawryszyn, P. Sauter, M. B. Biskup, U. Schepers, and S. Bräse, "Chemical Synthesis of Glycosaminoglycans," jul 2016.
- [133] E. Grand, J. Kovensky, G. Pourceau, S. Toumieux, and A. Wadouachi, "Anionic oligosaccharides: synthesis and applications," *Carbohydr. Chem*, vol. 40, pp. 195–235, 2014.
- [134] M. Bernfield, M. Götte, P. W. Park, O. Reizes, M. L. Fitzgerald, J. Lincecum, and M. Zako, "Functions of cell surface heparan sulfate proteoglycans," *Annual review of biochemistry*, vol. 68, no. 1, pp. 729–777, 1999.
- [135] K. Osterloh, U. Ewert, and A. R. Pries, "Interaction of albumin with the endothelial cell surface," *American Journal of Physiology-Heart and Circulatory Physiology*, vol. 283, no. 1, pp. H398–H405, 2002.

- [136] B. K. S. Yeung, P. Y. C. Chong, and P. A. Petillo, "Synthesis of glycosaminoglycans," *Journal of carbohydrate chemistry*, vol. 21, no. 7-9, pp. 799–865, 2002.
- [137] L. Schaefer and R. M. Schaefer, "Proteoglycans: from structural compounds to signaling molecules," *Cell and tissue research*, vol. 339, no. 1, p. 237, 2010.
- [138] T. C. Laurent and J. R. Fraser, "Hyaluronan.," *The FASEB journal*, vol. 6, no. 7, pp. 2397–2404, 1992.
- [139] S. Weinbaum, J. M. Tarbell, and E. R. Damiano, "The structure and function of the endothelial glycocalyx layer," *Annu. Rev. Biomed. Eng.*, vol. 9, pp. 121–167, 2007.
- [140] P. Prehm, "Hyaluronate is synthesized at plasma membranes," *Biochemical Journal*, vol. 220, no. 2, pp. 597–600, 1984.
- [141] N. Itano, T. Sawai, M. Yoshida, P. Lenas, Y. Yamada, M. Imagawa, T. Shinomura, M. Hamaguchi, Y. Yoshida, and Y. Ohnuki, "Three isoforms of mammalian hyaluronan synthases have distinct enzymatic properties," *Journal of Biological Chemistry*, vol. 274, no. 35, pp. 25085–25092, 1999.
- [142] D. Jiang, J. Liang, and P. W. Noble, "Hyaluronan as an immune regulator in human diseases," *Physiological reviews*, vol. 91, no. 1, pp. 221–264, 2011.
- [143] V. Bourguignon and B. Flamion, "Respective roles of hyaluronidases 1 and 2 in endogenous hyaluronan turnover," *The FASEB Journal*, vol. 30, no. 6, pp. 2108–2114, 2016.
- [144] J. R. F. Fraser, T. C. Laurent, H. Pertoft, and E. Baxter, "Plasma clearance, tissue distribution and metabolism of hyaluronic acid injected intravenously in the rabbit," *Biochemical Journal*, vol. 200, no. 2, pp. 415–424, 1981.
- [145] L. Lapčák, L. Lapcik, S. De Smedt, J. Demeester, and P. Chabreck, "Hyaluronan: preparation, structure, properties, and applications," *Chemical reviews*, vol. 98, no. 8, pp. 2663–2684, 1998.

- [146] F. E. Kendall, M. Heidelberger, and M. H. Dawson, "A serologically inactive polysaccharide elaborated by mucoid strains of group A hemolytic streptococcus," *Journal of Biological Chemistry*, vol. 118, no. 1, pp. 61–69, 1937.
- [147] V. N. Khabarov, P. Y. Boykov, and M. A. Selyanin, *Hyaluronic Acid: Production, Properties, Application in Biology and Medicine*. John Wiley & Sons, 2014.
- [148] R. C. Condie and G. D. Prestwich, "Engineering Clinically Useful Hyaluronan Matrices in Injectable Biomaterials: Science and Application; Vernon, B., Ed," 2010.
- [149] F. Duranti, G. Salti, B. Bovani, M. Calandra, and M. L. Rosati, "Injectable hyaluronic acid gel for soft tissue augmentation. A clinical and histological study.," *Dermatologic surgery : official publication for American Society for Dermatologic Surgery [et al.]*, vol. 24, pp. 1317–1325, dec 1998.
- [150] M. Averbek, C. A. Gebhardt, S. Voigt, S. Beilharz, U. Anderegg, C. C. Termeer, J. P. Sleeman, and J. C. Simon, "Differential regulation of hyaluronan metabolism in the epidermal and dermal compartments of human skin by UVB irradiation," *Journal of Investigative Dermatology*, vol. 127, no. 3, pp. 687–697, 2007.
- [151] E. A. Turley, P. W. Noble, and L. Y. W. Bourguignon, "Signaling properties of hyaluronan receptors," *Journal of Biological Chemistry*, vol. 277, no. 7, pp. 4589–4592, 2002.
- [152] S. A. Meyers, A. V. Seaber, R. R. Glisson, and J. A. Nunley, "Effect of hyaluronic acid/chondroitin sulfate on healing of full-thickness tendon lacerations in rabbits," *Journal of orthopaedic research*, vol. 7, no. 5, pp. 683–689, 1989.
- [153] D. A. R. Burd, M. Ritz, S. Regauer, M. T. Longaker, J. W. Siebert, and H. Garg, "Hyaluronan and wound healing: a new perspective," *British journal of plastic surgery*, vol. 44, no. 8, pp. 579–584, 1991.
- [154] K. R. Kirker, Y. Luo, J. H. Nielson, J. Shelby, and G. D. Prestwich, "Glycosaminoglycan hydrogel films as bio-interactive dressings for wound healing," *Biomaterials*, vol. 23, no. 17, pp. 3661–3671, 2002.

- [155] P. H. Weigel, G. M. Fuller, and R. D. LeBoeuf, "A model for the role of hyaluronic acid and fibrin in the early events during the inflammatory response and wound healing," *Journal of theoretical biology*, vol. 119, no. 2, pp. 219–234, 1986.
- [156] J. M. Estes, N. S. Adzick, M. R. Harrison, M. T. Longaker, and R. Stern, "Hyaluronate metabolism undergoes an ontogenic transition during fetal development: implications for scar-free wound healing," *Journal of pediatric surgery*, vol. 28, no. 10, pp. 1227–1231, 1993.
- [157] K. Rolfe and A. Grobbelaar, "A review of fetal scarless healing," *ISRN dermatology*, vol. 2012, 2012.
- [158] "Research Grade Sodium Hyaluronate - Lifecore. Retrieved from <https://www.lifecore.com/sodium-hyaluronate/research-grade-sodium-hyaluronate/>. Access date: 2019-06-11."
- [159] T. N. Snyder, K. Madhavan, M. Intrator, R. C. Dregalla, and D. Park, "A fibrin/hyaluronic acid hydrogel for the delivery of mesenchymal stem cells and potential for articular cartilage repair," *Journal of biological engineering*, vol. 8, no. 1, p. 10, 2014.
- [160] G. D. Prestwich, "Biomaterials from chemically-modified hyaluronan," *Glycoforum* <http://glycoforum.gr.jp/science/hyaluronan/HA18/HA18E.html>, 2001.
- [161] J. A. Burdick and G. D. Prestwich, "Hyaluronic acid hydrogels for biomedical applications," *Advanced materials*, vol. 23, no. 12, pp. H41–H56, 2011.
- [162] D. P. Nair, M. Podgórski, S. Chatani, T. Gong, W. Xi, C. R. Fenoli, and C. N. Bowman, *The Thiol-Michael addition click reaction: A powerful and widely used tool in materials chemistry*, vol. 26. American Chemical Society, 2014.
- [163] C. A. DeForest, B. D. Polizzotti, and K. S. Anseth, "Sequential click reactions for synthesizing and patterning three-dimensional cell microenvironments," *Nature materials*, vol. 8, no. 8, p. 659, 2009.
- [164] C. E. Hoyle and C. N. Bowman, "Thiol–ene click chemistry," *Angewandte Chemie International Edition*, vol. 49, no. 9, pp. 1540–1573, 2010.

- [165] R. A. Peattie, E. R. Rieke, E. M. Hewett, R. J. Fisher, X. Z. Shu, and G. D. Prestwich, "Dual growth factor-induced angiogenesis in vivo using hyaluronan hydrogel implants," *Biomaterials*, vol. 27, no. 9, pp. 1868–1875, 2006.
- [166] I. Gibas and H. Janik, "Synthetic polymer hydrogels for biomedical applications," 2010.
- [167] A. M. Kasko, "Degradable Poly(ethylene glycol) Hydrogels for 2D and 3D Cell Culture," *Materials Matters*, vol. 6, no. 3, pp. 67–75, 2011.
- [168] N. A. Peppas, K. B. Keys, M. Torres-Lugo, and A. M. Lowman, "Poly (ethylene glycol)-containing hydrogels in drug delivery," *Journal of controlled release*, vol. 62, no. 1-2, pp. 81–87, 1999.
- [169] "Michael addition polymerization. Retrieved from: http://polymerdatabase.com/polymer_chemistry/Michael_Addition.html. Access date: 2019-09-10."
- [170] J. L. Vanderhooft, M. Alcoutlabi, J. J. Magda, and G. D. Prestwich, "Rheological Properties of Cross-Linked Hyaluronan–Gelatin Hydrogels for Tissue Engineering," *Macromolecular bioscience*, vol. 9, no. 1, pp. 20–28, 2009.
- [171] E. A. BIO - Division of BioTime, "HyStem ® Hydrogels Cellular Matrices for Translational Research," tech. rep.
- [172] "BioTime Submits CE Mark Application for European Approval of Renevia® | Business Wire. Retrieved from <https://www.businesswire.com/news/home/20180313005625/en/BioTime-Submits-CE-Mark-Application-European-Approval>. Access date: 2019-09-09."
- [173] S. H. Mathes, H. Ruffner, and U. Graf-Hausner, "The use of skin models in drug development," *Advanced drug delivery reviews*, vol. 69, pp. 81–102, 2014.
- [174] T. A. Ahmed, E. V. Dare, and M. Hincke, "Fibrin: a versatile scaffold for tissue engineering applications," *Tissue Engineering Part B: Reviews*, vol. 14, no. 2, pp. 199–215, 2008.

- [175] M. G. Haugh, S. D. Thorpe, T. Vinardell, C. T. Buckley, and D. J. Kelly, “The application of plastic compression to modulate fibrin hydrogel mechanical properties,” *Journal of the mechanical behavior of biomedical materials*, vol. 16, pp. 66–72, 2012.

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2.1 Introduction

This chapter is devoted to the study and characterisation of human plasma-derived hydrogels containing 1.2 mg/ml and 2.4 mg/ml of final fibrin concentration. These numbers arise as two potentially optimal final fibrin concentrations due to the limited range available in human plasma samples. The objective of the experiments performed was to determine the optimal final fibrin concentration for designing a functional skin dermo-epidermal equivalent. Hydrogels designed using commercially available human fibrinogen have always used high levels of final fibrin concentration (5-20 mg/ml) in order to provide the hydrogels with the desired mechanical properties [1]. The absence of structurally supporting substances in commercial fibrinogen matrices has lead these researchers to increase the final fibrin concentration in the matrices to levels above the ones found in human blood [2].

In this thesis, we propose the use of whole blood plasma as a mean of better mimicking the environment cells encounter upon skin tissue damage. That decision has allowed us to use fibrin concentrations in line of what it is found *in vivo* conditions. Mechanical properties for hydrogels derived from plasma greatly outperform the commercial fibrinogen hydrogels of comparable final fibrin concentrations. This is due to the presence of several components in the human blood plasma like proteins and lipids, which confer better stability to the constructs. Still, a fibrin concentration that allows the cells to expand and proliferate needs to be selected for the correct design of the dermal equivalent. In that way a series of experiments that characterise the behaviour of plasma-derived hydrogels in terms of gelation time, mechanical properties, contractile behaviour and biological performance have been performed with the aim of selecting the appropriate platform upon which develop an optimal solution for

skin burns, chronic ulcers and other conditions.

2.2 Materials & Methods

2.2.1 Materials

Frozen human plasmas with low content of platelets were obtained from a blood bank in Spain (Banco de Sangre del Centro Comunitario de Transfusión del Principado de Asturias (CCST)) and treated according to the standards of the American Association of Blood Banks [3]. Antifibrinolytic agent Amchafibrin (Tranexamic acid 500mg, Meda Pharma SL, Spain), sodium chloride (NaCl 0.9%) and calcium chloride (CaCl_2 1%) (Sigma Aldrich, USA) were used for hydrogels formation. Phosphate-buffered saline (PBS) (Sigma Aldrich, USA) was used to incubate hydrogels. Bradford assay reagent (Bio-Rad Protein Assay Dye Reagent Concentrate) (Bio-Rad, Germany) and Bovine serum albumin (BSA) (1 mg/ml) (Sigma Aldrich, USA) were used to measure protein release. Different dilutions of pure ethanol were used to dehydrate hydrogels for structural characterisation. As cell culture medium, Dulbecco's Modified Eagle's Medium (DMEM) + GlutaMAXTM supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin (P/S) (Thermo Fisher Scientific, USA) were used. Cellular proliferation and viability was measured with Alamar BlueTM Cell Viability Reagent and Ibidi[®] μ -slide 8 well glass bottom were used for confocal microscopy visualization of hydrogels in the Live/Dead[®] Cell Assay (Thermo Fisher Scientific, USA). Keratinocyte proliferation was measured in special medium CNT57 obtained from Cellntec, Spain. MTS Reagent for cell viability assay was purchased to Sigma Aldrich, USA.

2.2.2 Plasma-derived fibrin hydrogels preparation

Plasma-derived hydrogels were prepared following the protocol previously described by Meana *et al.* [4]. Human platelet-poor plasma was obtained from a blood bank (Banco de Sangre del Centro Comunitario de Transfusión del Principado de Asturias), was stored at -80°C upon arrival to the facility and was defrosted prior to its use.

Preparation of the hydrogels was performed as follows: plasma of known

fibrinogen concentration and from the same batch was defrosted by putting the aliquots in a pre-warmed water bath at 37°C. Calculations were made to know the volume needed of plasma to obtain hydrogels with a final fibrinogen concentration of both 1.2 mg/ml and 2.4 mg/ml. After the human plasma was added to the appropriate recipient (glass-vial, culture plate, falcon tube), tranexamic acid (AmchaFibrin 500mg, Meda Pharma SL, Spain), an antifibrinolytic agent was added to reach a final concentration of 0.08% w/v. Furthermore, when hydrogels contained human primary fibroblast, these were always added in a fixed volume of 40 µl for every millilitre of hydrogel. Finally, in order to trigger the coagulation cascade, calcium chloride (CaCl₂ 1%) previously prepared in sodium chloride (NaCl 0.9%) and sterile filtered was added to a final concentration of 0.08% in weight/volume ratio for the 1.2 mg/ml hydrogels and of 0.16% w/v for the hydrogels at 2.4 mg/ml final fibrin concentration. The mixture was adjusted to the final desired volume by adding the corresponding volume of sodium chloride (NaCl 0.9%) prepared in deionised water (dH₂O) and sterile filtered prior to its use. Plasma hydrogels components were added in the following order: human plasma, amchafibrin, NaCl 0.9%, human primary fibroblast and CaCl₂ 1%.

Human plasma hydrogels mixtures were then incubated for 1 hour at 37°C in an atmosphere containing 5% CO₂ and 40% relative humidity for their gelation. Upon complete gelation and depending on the experiment carried out, plasma hydrogels had to be detached from their container. In order to do that, pre-warmed PBS at 37°C was used to remove the gels from vials by adding it against the glass wall until complete detachment was achieved. In this case, plasma hydrogels were transferred to the appropriate recipient to perform the experiments (p35 culture plates or p60 culture plates).

2.2.3 Gelation time and kinetics

Human plasma-derived hydrogel gelation time was determined using the flip-flop method [5, 6]. This method is based on the free flowing of liquid inside the vials where hydrogels were prepared. Glass vials containing the hydrogels mixture were tilted every 30 seconds to accurately determine the time in took for the mixture solution to stop freely flowing inside the vials. Plasma hydrogels were first prepared under laminar-flow hood and later incubated at 37°C, 5% CO₂ and 40% relative humidity for their gelation. Final gelation time was

assessed when no liquid was left in the vial and the hydrogel was stuck in the bottom.

Further characterisation of the gelation process was achieved through the study of the gelation kinetics with UV turbidimetry assay. The gelation kinetics were monitored using a SynergyTM HTX Multi-Mode Microplate Reader (Winooski, VT, USA). Hydrogels solutions were placed into a solid glass 96-well plate (6.4 mm diameter, 0.32 cm² area) and quickly transferred to the microplate reader for their measurement. A glass system was used to ensure the proper gelation of the plasma hydrogels in the absence of human primary fibroblast, by using a negatively charged surface the coagulation cascade was promoted by activation of Factor XIII [7].

In order to accurately measure the gelation kinetics, first the UV-wavelength at which the solution to gel transitions could be measured was determined. Both a hydrogel solution not capable of forming a gel (lacking CaCl₂) and the fully gelled hydrogel were subjected to a UV scan measurement ranging from 200 nm to 800 nm in 5 nm steps. In this way, the UV-wavelength was determined by selecting the point in which the difference in O.D. between the mixture solution and the hydrogel was the maximum, in our case 325 nm.

Once the optimal UV-wavelength was assessed, the UV gelation experiment was performed by incubating the hydrogels solutions at 37°C for 1 hour with absorbance reading intervals of 30 seconds to accurately characterise the gelation kinetics of the hydrogels.

2.2.4 Hydrogel contraction

Weight contraction

Human plasma-derived hydrogels contraction was measured upon the differences in weight recorded with a precision balance after different time intervals of incubation in PBS. This experiment was performed using 19 mm diameter glass vials where the hydrogels were made and p35 culture dishes for the incubation period. The first step was weighting all this material, both the glass vials and the culture dishes, in a precision scale. Plasma hydrogels were then made as explained in section 2.2.2 and left in the incubator at 37°C, 5% CO₂

and 40% relative humidity for 1 hour. After this period of time glass vials containing the hydrogels stuck in the bottom were weighted to determine the initial weight (W_0) of the system.

Plasma hydrogels at both 1.2 mg/ml and 2.4 mg/ml of fibrinogen concentration needed to be detached from the vials and transferred to p35 culture dishes in order to be incubated in a 3D environment totally submerged in PBS. Hydrogels were detached from the glass vials following always the same procedure (Figure 2.1). Pre-warmed PBS at 37°C was added against the wall of the vials until the gel was completely detached. Then, hydrogels were ready to be transferred to the p35 culture dishes and submerged in 3ml of PBS. Hydrogels were left incubating at 37°C between time point intervals (0h, 1h, 2h, 4h, 6h, 8h, 24h, 48h and 72h).

Figure 2.1: Detachment method for the hydrogels prepared in glass vials using PBS at 37°C

Weight differences were measured at each time point (0h, 1h, 2h, 4h, 6h, 8h, 24h, 48h and 72h) carefully drying the culture plate by removing the PBS and then drying the surface with cotton swabs. Plasma hydrogels were measured in a precision scale and its weight annotated (W_T). Swelling ratio of the hydrogels was then calculated with the following equation 2.1.

$$SR_W = \frac{W_T}{W_0} \quad (2.1)$$

Being SR_W the swelling ratio in weight, W_T the weight at each time-point and W_0 the initial weight of the hydrogel. After each of the measurements, new fresh 3ml of PBS was added to the p35 dish in order for the contraction experiment to continue.

Area contraction

In combination with the weight contraction experiment, the difference in surface area of the hydrogels during incubation in PBS at 37°C was also measured. Contraction in the XY plane was computed through the analysis of images taken at the same time point as the weight study (0h, 1h, 2h, 4h, 6h, 8h, 24h, 48h and 72h). Images were taken with a smartphone camera with plasma hydrogels over a matte dark background and with a 30cm ruler that acted as scale.

Images were then analysed with the image processing software Image J (Figure 2.2). First, pixel to cm ratio was measured using the 30cm ruler placed next to the p35 culture dish. Once the image was converted to the metric unit system, the colour threshold tool was used in order to discern the hydrogel from the background. Finally, the tool “analyse particles” was used in order to measure the area of the hydrogels. Size range was set between 2 and 5 cm² and circularity between 0.75 and 1.00 so that the hydrogel was correctly selected.

Swelling area ratio was calculated similarly to the weight ratio using the following equation 2.2:

$$SR_A = \frac{A_T}{A_0} \quad (2.2)$$

Being SR_A the swelling ratio in area, A_T the surface area at each time-point and A_0 the initial area of the hydrogel. Plasma hydrogels were maintained at 37°C after each picture was taken in order to complete the experiment.

Figure 2.2: Screenshot of the software (ImageJ) and method used to quantify the area change in hydrogels.

2.2.5 Protein release

Human plasma hydrogels contain a varied set of proteins in their structure, mainly albumin but also different immunoglobulins. The cumulative release of these proteins from the hydrogels when incubated in PBS at 37°C was measured through the classical Bradford assay first described in 1976 [8]. This assay is based on the change in colour and absorbance of Coomassie Brilliant blue G-250 in the presence of proteins. When no proteins are present in the mixture, Coomassie G-250 presents a brown colour in its acidic form; while when proteins are present, a change in its absorbance from 465 nm to 595 nm occurs, making

also a shift in its colour from brown to blue (Figure 2.3). This absorbance at 595 nm can be then measured in the spectrophotometer and its value related to protein concentration by extrapolating the data from a standard curve.

Figure 2.3: Working principle for the Coomassie Brilliant blue G-250 [9].

The first step was to make a standard curve with known concentrations of bovine serum albumin (BSA). Seven different BSA concentrations were used in order to produce the standard curve. The stock concentration of BSA was 1 mg/ml and the final volume of the mixtures was 1ml. The following table (2.1) shows the volumes of the reagents used and the final concentration of BSA for the conditions tried.

PBS	BSA (μ l)	Bradford reagent (μ l)	[BSA] (μ g/ml)
800	0	200	0
798.95	1.25	200	1.25
797.5	2.5	200	2.5
795	5	200	5
790	10	200	10
785	15	200	15
780	20	200	20

Table 2.1: Volumes and final concentration of BSA to produce the standard curve.

From the experimental data, the linear range of BSA concentrations (0-20 μ g/ml) was plotted (O.D. at 595 nm vs. BSA concentration) and the equation

of the line ($y = mx + c$) and R^2 coefficient were calculated.

Samples of 1ml of PBS were obtained from the surrounding medium in which plasma hydrogels were incubated at the designated time points (0h, 1h, 2h, 4h, 6h, 8h, 24h, 48h and 72h) and were diluted to be on the range of the standard curve (0-20 $\mu\text{g/ml}$). In order to do that, 5 μl of sample was diluted in fresh 795 μl of PBS and finally 200 μl of the Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad, USA) were added and the O.D. of the mixture measured at 595 nm in the Biowave II spectrophotometer (Biochrom, UK). Whenever the O.D. fell into the negative range, 20 μl of the sample were diluted into 780 μl of PBS and 200 μl of dye reagent in order to increase the sensibility of the system. All dilution factors were taken into account to calculate the final concentration and total protein released from the plasma hydrogels.

Cumulative protein release was calculated by multiplying the protein concentration at each time point by the total amount of PBS (3ml) and adding each time point to reach the total amount released at every time.

2.2.6 Mechanical characterization: compression test

Plasma hydrogels at both final fibrin concentrations of 1.2 mg/ml and 2.4 mg/ml were subjected to a classical compression test to compare their biomechanical properties. The test was performed at Instituto de Ciencia y Tecnología de polímeros (ICTP) that belongs to the Consejo Superior de Investigaciones Científicas (CSIC).

The machine used to test the samples was an electromechanical system Instron 3366 and the type of test performed was an uniaxial compression test. Two parallel plates serve as the platform to load the samples. The upper plate is connected to the load cell (100 N) and is the mobile part that will compress the hydrogels against the lower plate (Figure 2.4). The whole system is connected to the computer where software Bluehill 3 serves as the controlling unit. A protocol was defined inside the software to account for the initial ($d_0=25$ mm) and final diameter of the specimens tested as well as the height of the hydrogels in order to compute the stress and strain values. The compression rate was set at 2 mm/sec.

Figure 2.4: Instron 3366 and Bluehill3 software. Adapted from [10].

Hydrogels were prepared in 25 mm diameter glass vials and detached and left incubating at 37°C in PBS for 24 hours prior to testing to reach equilibrium. The final volume of the samples was set to 5ml, giving an initial height of ≈ 1 cm. Before the testing was performed, hydrogels were carefully dried removing the PBS and using cotton swabs to fully dry the surface. Samples were then placed over the lower plate of the compression machine and its experimental height measured by placing the upper plate just in contact with its surface. This number was annotated and introduced into the software in order to correctly calculate the strain.

The results recorded by the compression machine were displayed as load in Newtons (N) and strain in percentage (%). The data was then used to calculate the engineering stress (σ), engineering strain (ϵ) of the samples tested with the following equations 2.3 and 2.4:

$$\sigma = \frac{F}{A_0} \quad (2.3)$$

$$\epsilon = \frac{\Delta L}{L_0} \quad (2.4)$$

Elastic materials like these hydrogels show this behaviour at strains below 20%. The compressive modulus of elasticity or Young's Modulus (E) was calculated taking this 20% point of strain with equation 2.5:

$$E = \frac{\sigma(\varepsilon)}{\varepsilon} = \frac{F/A}{\Delta L/L_0} \quad (2.5)$$

Being F the force in Newtons, ΔL the increment in length, A_0 the initial area and L_0 the initial length. Results for the compression test were displayed with the stress-strain curve and the compressive modulus of elasticity in order to compare the two types of hydrogels tested (1.2 mg/ml and 2.4 mg/ml).

2.2.7 Structural characterisation (SEM)

Human plasma-derived hydrogels were structurally characterised through scanning electron microscopy (SEM). In order to maintain its internal morphology, samples were subjected to supercritical CO₂ point drying. This technique is based on the change of state of CO₂ from liquid to gas without change in volume. The point at which this phenomenon occurs is called critical point and for CO₂ is known to be 31.1°C of temperature and 73.9 bar of pressure. Above this temperature and pressure, CO₂ enters in a state called supercritical fluid, in which its properties are a density like in liquid state but occupying the maximum volume available (Figure 2.5). The advantage of this technique compared to lyophilisation is that it maintains the internal structure when drying the sample.

Samples for structural characterisation were prepared three days prior to their drying with the supercritical reactor. This time was allowed for the exchange of ethanol with water to occur. This step is crucial since water is immiscible with CO₂ hindering its application to the critical point drying procedure. Instead, water is substituted with ethanol in a progressive manner so that now ethanol will be dissolved in liquid CO₂ allowing for the exchange of these liquids inside the supercritical reactor. First, five different dilutions of water and ethanol containing percentage ranges of ethanol of 20%, 40%, 60%,

Figure 2.5: CO₂ phase diagram [11].

80% and 100% were prepared. Then, plasma hydrogels were prepared inside 19 mm diameter glass vials with a final volume of 3ml. After 1 hour of gelation, hydrogels were detached with pre-warmed PBS as explained in section 2.2.4 and transferred to 50ml falcon tube containing the first 20% ethanol dilution. The following table 2.2 resumes the procedure followed in order to exchange water with ethanol.

Ethanol (%)	Incubation time (h)
20	2
40	2
60	12
80	2
100	12

Table 2.2: Ethanol-water exchange conditions.

Once the dehydration of samples was performed, hydrogels were kept in 100% ethanol and brought to CSIC for their critical point drying. Thar Technologies supercritical fluid reactor (Figure 2.6) was used to remove the ethanol by supplying a constant flow of supercritical CO₂ inside the reactor and then

depressurising the chamber in order for the CO₂ to evaporate without changing its volume. The conditions used inside the reactor are resumed in table 2.3.

Time (min)	Temperature (°C)	Pressure (bar)
10	35	0-100
90	35	100
30	35	100-0

Table 2.3: Supercritical CO₂ drying conditions.

Figure 2.6: Supercritical CO₂ reactor [12].

Hydrogels came off the reactor as a white dried disc and high porosity structure. In order to visualize them in the SEM, clean sectioning had to be performed. Samples were submerged in liquid nitrogen until they froze and a clean cut was performed with the aid of a pair of tweezers. Cross-section of hydrogels were mounted in the SEM holder and gold-sputtered for 75 seconds in Leica ACE600. This process will confer the samples the conductivity needed for SEM visualization as well as enhance the topographical contrast [13]. The final step was to bring the mounted sample into the Philips XL30 SEM (Figure 2.7) at

the Materials Science and Engineering Department at Universidad Carlos III de Madrid (UC3M) and take the images at different magnifications (5,000X, 10,000X and 20,000X). The conditions used were the secondary detector with a current voltage of 15kV. Images were taken and analysed for fibre width with ImageJ software.

Figure 2.7: Scanning electron microscope Philips XL-30 [14].

2.2.8 Cell culture

Human primary fibroblast obtained from skin biopsies were used to perform the cellular experiments involving the encapsulation of cells inside the plasma-derived fibrin matrices. Fibroblasts were stored in liquid nitrogen at -195.8°C and defrost and seeded into T75 flask before the start of each experiment. Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin/Streptomycin (P/S) antibiotic/antimycotic agent was used as culture medium. Cells were maintained at 37°C and 5% CO_2 pressure in the incubator and media was changed every 2 days until fibroblast reached confluence.

Detachment from the T75 flask proceeded as follows: pre-warmed PBS was

added into the culture flask after medium had been vacuumed in order to remove any excess of supplemented DMEM. Two millilitres of trypsin were added into the flask and left incubation for 10 minutes at 37°C in order to detach the cells from the bottom. The flask was visualised under the microscope to assure all the fibroblast were floating, then trypsin was inactivated by adding DMEM supplemented with 10% fetal bovine serum (FBS) and 1% (P/S) culture medium on top. Fibroblast were then counted by putting 10 µl of the solution into a Neubauer chamber and counting under the microscope in order to assess the number of cells. The solution containing the fibroblast was then centrifuged for 7 minutes at 1200 rpm after which the supernatant was discarded and the pellet of cells was re-suspended in the desired amount of supplemented medium to perform the experiment.

In order to generate the dermo-epidermal skin constructs, human primary keratinocytes were used on top of the constructs to form the epidermal layer. This type of cells are incapable of growing *in vitro* in the absence of a feeder layer of cells that in this case was conformed by irradiated mouse embryonic fibroblast (3T3 ± J2/Swiss albino mouse 3T3). This irradiated layer of cells is incapable of proliferating thus allowing the primary keratinocytes to form colonies and grow using the factors generated by the feeder cells. The culture medium used for keratinocytes contained a 3:1 mixture of DMEM and HAM's F12, supplemented with 10% FBS, 8 ng/ml choleric toxin, 2 nM T3, 0.4 mg/ml hydrocortisone, 5 mg/ml insulin, 10 ng/ml EGF and 25 µg/ml adenine.

Human primary keratinocytes were detached from the T75 flask following the previously explained protocol with a minor change in terms of trypsin incubation that changed to 20 minutes at 37°C.

2.2.9 Fibroblast-mediated matrix contraction

Human plasma hydrogels contraction in the presence of human primary fibroblast was studied through section area change at different time points (0h, 4h, 6h, 8h, 24h, 48h, 72h, 7 days, 10 days and 15 days). Hydrogels preparation protocol was changed to accommodate the inclusion of human primary fibroblast as explained in section 2.2.2. Cell density was set to 80,000 cells/ml of hydrogels prepared. Once both plasma 1.2 mg/ml and 2.4 mg/ml hydrogels were prepared in 19 mm diameter glass vials, they were left incubating for full

gelation for 1 hour at 37°C. After this period of time, hydrogels containing fibroblast were detached with pre-warmed DMEM supplemented with 10% FBS and 1% P/S by following the procedure in section 2.2.4 (Figure 2.1).

Images were taken of the gels at 0h with a black matte background and a ruler acting as scale for later image processing. For the complete duration of the experiments plasma hydrogels were kept at 37°C, 5% CO₂ and 40% relative humidity. Samples were taken off the incubator and a new image taken for each of the time points (0h, 4h, 6h, 8h, 24h, 48h, 72h, 7 days, 10 days and 15 days) and media was changed every two days. Swelling area ratio was calculated with equation 2.2.

2.2.10 Fibroblast proliferation assay

Human primary fibroblast proliferation inside the plasma hydrogels was studied through the Alamar BlueTM assay. This assay is based on the reduction of resazurin, a non-toxic, membrane permeable compound that has no fluorescence activity, into resorufin, a fluorescent compound that can be measured in the microplate reader. In this way, proliferation activity of cells incorporated to the hydrogels was measured indicating the cell viability [15].

Hydrogels were prepared with a final volume of 100 µl in Costar[®] Black 96-well plates with 6 replicates per condition. Human primary fibroblasts were incorporated into the hydrogels solution at a density of 80,000 cells/ml of gel. Samples were left for gelation for 1 hour at 37°C and after this period of time 150 µl of supplemented medium was added on top of each gel. Four different plates were prepared with hydrogels containing 1.2 mg/ml and 2.4 mg/ml of final fibrin concentration, each plate was labelled with the time point in which it would be measured (1 day, 3 days, 7 days and 14 days).

At every time point, the plate was taken out of the incubator and the medium removed from each gel with a pipette. Alamar BlueTM cell viability reagent was diluted in supplemented medium to a 10% concentration and 100 µl of the solution were added to each well. The plate was then incubated for 3 hours at 37°C and 5% CO₂. After the incubation time with the reagent, 100 µl of the medium on top of the gels was transferred to the lower wells of the 96-well plate in order to measure its fluorescence in the SynergyTM HTX

Multi-Mode Microplate Reader. Fluorescence was measured at 570 nm excitation wavelength and 600 nm emission wavelength and the fluorescence intensity was plotted (Figure 2.8).

Figure 2.8: Alamar BlueTM assay protocol [15].

2.2.11 Live/Dead assay

Human primary fibroblast viability inside both concentration hydrogels was further characterised with the classical Live/Dead[®] Viability/Cytotoxicity kit for mammalian cells assay. This assay is based on the simultaneous detection of live and dead cells with two different dyes, calcein AM and ethidium homodimer. The detection of live cells is based on the esterase activity produced inside viable cells in culture, making the change from the non fluorescent product calcein AM into a bright green fluorescence calcein which can be detected with 495 nm excitation and 515 nm emission filter in the SynergyTM HTX Multi-Mode Microplate Reader. On the other hand, dead cells are detected upon the membrane permeability of cells when they die. Ethidium homodimer (EthD-1) is capable of entering the disrupted membrane of non-viable cells and increase its fluorescence by forty times when bond to the nucleic acids producing an intense red fluorescence detectable at 530 nm excitation and 645 nm emission spectrum.

Hydrogels were prepared in μ -slide 8-well glass bottom plates purchased from Ibidi[®] so that confocal microscopy could be performed as the hydrogels are a 3D culture platform. Final volume of plasma hydrogels at 1.2 mg/ml and 2.4 mg/ml fibrin concentration was set to 100 μ l in order to have an approxi-

mate height of 2 mm. A total of 16,000 cells per well or 160,000 cells/ml were studied for each condition. Gelation was allowed to develop for 1 hour at 37°C and 5% CO₂ before adding 200 µl of cell culture medium containing 10% FBS and 1% P/S. Hydrogels were left incubating for 48 hours before performing the Live/Dead assay.

The day of the confocal microscope reservation, µ-slide 8-well glass bottom plate was taken off the incubator and the medium removed. Wells were washed with PBS two times for 5 minutes before adding the assay reagents. PBS solution of the desired amount (200 µl/well) was prepared containing calcein AM at a concentration of 0.5 µl/ml of PBS and ethidium homodimer with final concentration of 2 µl/ml of PBS. This solution was added to the medium-less wells containing the hydrogels and fibroblast and wrapped in aluminium foil. The plate was incubated for 30 minutes at 37°C. After the incubation period, Live/Dead reagents were removed and the excess of material was washed out by adding PBS three times for 5 minutes. Once the washing was performed, the plate was covered with aluminium foil and brought to the Fundación Jiménez Díaz for its visual characterisation in the confocal microscope.

Cell viability was calculated by counting the viable cells with ImageJ and dividing them by the number of dead cells, obtaining a percentage (%) of viability ratio.

2.2.12 Keratinocyte proliferation assay (MTS)

Human primary keratinocytes were used in combination with the hydrogels system in order to create a full thickness skin culture model. Keratinocytes were first expanded in co-culture with irradiated mouse fibroblast that acted as a feeder layer. Once the keratinocytes reached full confluence they were ready to be used. The ability of primary keratinocytes to attach to the hydrogels surface and proliferate to form a monolayer was assessed with the MTS assay. MTS is based on the change in absorbance of tetrazolium compound when live cells reduce this product to generate formazan dye that can be measured in absorbance at 490 nm in the microplate reader. Reduction of this product in metabolically active cells is mediated by the NAD(P)H-dependent dehydrogenase enzymes.

Hydrogels samples were prepared in 96-well plates in the absence of human fibroblast due to its potential effect on the reduction of the reagent. In order for the keratinocytes that where seeded on top at a density of 100,000 cells/ml of hydrogel to proliferate, special conditioned medium (CNT57) was used. Plasma hydrogels final volume was set to 100 μ l and gelation time was left to proceed for 2 hours to ensure that gels would correctly gelify in the absence of fibroblasts. After this step, 100 μ l of conditioned medium was added for 4 hours in order to eliminate the excess of calcium inside the hydrogels. Finally, human primary keratinocytes were seeded on top and the constructs left for 48 hours untouched at 37°C, 5% CO₂ and 40% relative humidity.

After the 48 hours, the primary keratinocytes were attached to the top of the hydrogels and this time was considered as day 0. Three plates had been prepared each for every time point considered (1, 4 and 7 days). At each time point, medium was absorbed from each well and conditioned medium with a 10% MTS reagent was added. The plate was left for incubation for 2 hours at 37°C and 5% CO₂ before transferring the already reduced medium to a clean new well for each of the conditions. The plate was measured for O.D. at 490 nm at the SynergyTM HTX Multi-Mode Microplate Reader.

2.2.13 Contraction in organotypic skin constructs (z-axis)

In order to study the effect of both human primary fibroblast and human primary keratinocytes for the matrix contraction a new experiment was designed. Plasma hydrogels were prepared inside transwells in order to generate dermo-epidermal skin constructs *in vitro* following the protocol previously reported by Meana *et al.* [4]. Human primary fibroblasts were incorporated at a 20,000 cells/ml density and hydrogels were left incubating for 1 hour. Human primary keratinocytes were later seeded after 24 hours on top of the plasma hydrogels at a density of 1.5 million cells in 1 ml. Keratinocytes were left to sink and attach to the hydrogels surface for 48 hours before transferring the whole system into deepwell plates. Medium was then removed from the top of transwells in order for the keratinocyte differentiation to start in contact with the air. This air liquid interface served as the system to generate organotypic skin constructs.

Photographs were taken of the profile of the transwells in the presence of a ruler that acted as scale for the measurement of the height of the hydrogels

during the differentiation process. Defined time points were 0h, 24h, 48h, 72h, 7 days, 10 days, 15 days and 21 days. Images were analysed with ImageJ software.

2.2.14 Statistical analysis

Statistical analysis was performed using IBM[®] SPSS software. All the data averages and standard deviations were subjected to Student's t-test [16] in order to determine potential statistical difference significance in the results. Three levels of significance were assessed: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

2.3 Results

2.3.1 Gelation time and kinetics

Plasma-derived hydrogels gelation time was first assessed with the flip-flop method, selecting as final gelation time the point at which the mixture stop freely flowing inside 19 mm diameter glass vials. The results obtained for hydrogels containing final concentrations of fibrinogen of 1.2 mg/ml and 2.4 mg/ml showed a slight difference between the two conditions (Figure 2.9).

Hydrogels at 1.2 mg/ml of fibrinogen concentration stopped flowing inside the vials at 12 ± 1.4 minutes after mixing the components; while the hydrogels with final fibrinogen concentration of 2.4 mg/ml reached the same gel state after 14.5 ± 0.6 minutes. The difference found was not double even though the fibrin concentration was the double. The amount of CaCl_2 added to the more concentrated fibrinogen hydrogels was double that of the 1.2 mg/ml plasma gels.

Furthermore, the polymerization kinetics of fibrinogen into a fibrin network was studied through UV spectroscopy. In the transition from solution to gel state, a change in O.D. in the hydrogels can be measured giving information about the rate of polymerization and time to reach the hydrogel state, evidenced by a plateau in the O.D. readings. In order to correctly determine this transition for the plasma hydrogels, first a UV scan to find the wavelength at which this change in O.D. is best measured needed to be done. UV scan was performed over fully gelled hydrogels and over solutions incapable of forming a hydrogel to check the difference in O.D. at any given wavelength. The scan

Figure 2.9: Gelation times with the flip-flop method for plasma hydrogels at 1.2 mg/ml and 2.4 mg/ml final fibrin concentration.

was run from 200 nm to 800 nm giving as result a maximum difference of O.D. between the two different states at 325 nm (Figure 2.10).

This wavelength (325 nm) was selected to later perform the UV gelation kinetics experiment that consisted in measuring the O.D. dynamically every 30 seconds for 40 minutes. Hydrogels were quickly prepared and measured inside a pre-warmed to 37°C SynergyTM HTX Multi-Mode Microplate Reader. Results show the difference in O.D. between the more diluted plasma hydrogels (1.2 mg/ml) that reached a difference of 0.4 O.D. units between their solution and gel states and the more concentrated hydrogels (2.4 mg/ml) that obtained a maximum difference of 1 O.D between its two states (Figure 2.11).

2.3.2 Hydrogels contraction

Weight contraction

Plasma hydrogels with 1.2 mg/ml and 2.4 mg/ml final fibrin concentration were studied for weight contraction when incubated with PBS at 37°C. Initial hydrogels weight was determined shortly after gelation by weighting the p35 culture

Figure 2.10: UV scan of A) Plasma solution and hydrogel at 1.2 mg/ml final fibrin concentration B) Plasma solution and hydrogel at 2.4 mg/ml final fibrin concentration.

dish in the presence of the hydrogel in a precision scale. After this measurement, 3 ml of PBS were added and the plate incubated until the next time point. The results obtained for this experiment show that plasma hydrogels, regardless of their concentration contract in terms of weight when submerged in PBS (Figure 2.12). Most of the contraction, that reaches around 25% of the hydrogels weight, occurs during the first 6 hours of incubation. Both hydrogels showed the same level on contraction, indicating there are no differences

Figure 2.11: Gelation kinetics followed through UV spectroscopy and measured at 325 nm for plasma-derived hydrogels at 1.2 and 2.4 mg/ml final fibrin concentration.

between these two systems in terms of weight loss.

Figure 2.12: Contraction in weight at 37°C for hydrogels at 1.2 mg/ml and 2.4 mg/ml final fibrin concentrations.

Area contraction

Plasma hydrogels contraction was further assessed in terms of the surface area shrinking when incubated in PBS and 37°C. Pictures of the hydrogels at each time point (0h, 1h, 2h, 4h, 6h, 8h, 24h, 48h and 72h) were taken and later analysed with imaging processing software ImageJ. The contraction in the XY plane was expressed as a swelling ratio representing the total amount of surface area loss during the incubation. Results for both systems, 1.2 mg/ml and 2.4 mg/ml, show a decrease in area surface as time progresses, with an initial loss of 15% and 10% of surface area in the first hour, respectively (Figure 2.13). From that point on, hydrogels suffer barely any variation in their surface area, following a parallel trend maintaining the area lost to around 20% for 1.2 mg/ml hydrogels and around 15% for the 2.4 mg/ml hydrogels.

Figure 2.13: Area contraction at 37°C for hydrogels at 1.2 mg/ml and 2.4 mg/ml final fibrin concentrations.

2.3.3 Protein release

The plasma component of these hydrogels naturally contains a set of proteins well characterised and mainly dominated by albumin. This experiment was set to obtain a profile of the release of this set of proteins to the incubating

medium when immersed in PBS at 37°C. In order to do so, first a standard curve obtained with known concentrations of bovine serum albumin (BSA) was made (Figure 2.14). The following table resumes the values of O.D. obtained for each BSA concentration (Table 2.4).

[BSA] (µg/ml)	Optical Density (595 nm)
0	0
1.25	0.016
2.5	0.088
5	0.17
10	0.354
15	0.506
20	0.654
25	0.777

Table 2.4: Final concentration of BSA and O.D. values to generate the standard curve.

Figure 2.14: Bradford assay calibration curve.

Bradford assay consist in the change of colour and absorbance for the reagent added to the samples when this samples contain proteins. This absorbance can be measured in the UV-Vis spectroscopy machine and extrapolated to the

previously generated standard curve to quantify the total amount of proteins released. Plasma hydrogels with both final concentrations (1.2 mg/ml and 2.4 mg/ml) showed very different final amount of proteins released (Figure 2.15). The profiles of release were virtually equal with an initial burst of proteins in the first 2 hours and a progressive loss of proteins until reaching a plateau after 24 hours of incubation in PBS (Figure 2.15). Doubling the plasma content in the hydrogels systems has doubled the total amount of proteins that are released from the hydrogels.

Figure 2.15: Cumulative protein release at 37°C from the plasma-derived hydrogels at 1.2 mg/ml and 2.4 mg/ml final fibrin concentrations.

2.3.4 Mechanical characterisation: compression test

Plasma hydrogels at both final fibrin concentrations (1.2 mg/ml and 2.4 mg/ml) were subjected to a uniaxial compressive test to check their mechanical properties. The test was carried out in triplicates with samples of 5 ml final volume after a day of equilibration in PBS. Results were expressed in terms of the elastic modulus of compression or Young's modulus (E) calculated from the stress vs. strain curve at 20% strain (linear region) and the maximum load withstood by the hydrogels when subjected to 70% strain.

Results show that increasing the fibrin concentration from 1.2 mg/ml to 2.4 mg/ml indeed affects the mechanical properties by doubling the elastic moduli and the maximum load. Young's modulus value at 20% strain increased from 776 Pa to 1533 Pa, doubling the rigidity of the hydrogels (Figure 2.16A). In the same way, maximum load at 70% strain increased from 1.24 N to 2.02 N when the final fibrin concentration was doubled from 1.2 mg/ml to 2.4 mg/ml (Figure 2.16B).

Figure 2.16: Mechanical characterisation of plasma hydrogels A) Elastic modulus (Pa) of samples at 20% strain (linear region); B) Load (N) at 70% strain.

2.3.5 Structural characterisation (SEM)

Plasma hydrogels internal morphology was assessed through scanning electron microscopy (SEM). In order to dry the gels maintaining their internal structure, supercritical CO₂ point drying method was used. Hydrogels with final volume 3 ml with final fibrin concentrations of 1.2 mg/ml and 2.4 mg/ml were used to perform the study. Images obtained show the clear difference in structure and overall porosity when doubling the fibrin concentration (Figure 2.17). Hydrogels at 1.2 mg/ml of fibrin concentration showed smooth and intertwined fibres with a measured average fibre size of 170 nm. In the case of 2.4 mg/ml hydrogels fibres tend to aggregate forming a conglomerate structure where porosity decreases and no fibre diameter can be measured.

Figure 2.17: Representative images of plasma-derived hydrogels after 72 hours incubation at 37°C under SEM with A) 1.2 mg/ml fibrin concentration at 5,000X; B) 1.2 mg/ml fibrin concentration at 20,000X; C) 2.4 mg/ml fibrin concentration at 5,000X; D) 2.4 mg/ml fibrin concentration at 20,000X.

2.3.6 Fibroblast-mediated matrix contraction

The capacity of human primary fibroblasts to contract the hydrogels was studied through area change measured through photographs. Hydrogels were loaded with 80,000 cells/ml and prepared in 19 mm diameter glass vials. Time zero was set as the area occupied by the hydrogels after detachment and transference to p35 culture dishes. Plasma hydrogels at both concentrations (1.2 mg/ml and 2.4 mg/ml) were followed for 15 days in order to assess the effect of fibrin concentration on fibroblast elongation and contraction of hydrogels.

Results show that doubling the concentration of fibrin and thus making hydrogels less porous as evidenced by SEM images inhibits the contraction of hydrogels in the XY plane in terms of swelling ratio (Figure 2.18). Furthermore area change between both systems was calculated and statistical significant differences were found at 24, 48 and 72 hours time points evidencing the difference

in time that fibroblast take to fully expand and start contracting the matrix that surrounds them (Figure 2.19).

Figure 2.18: Fibroblast-mediated matrix contraction of plasma-derived hydrogels at 1.2 mg/ml and 2.4 mg/ml final fibrin concentrations.

Figure 2.19: Differences in area contracted (mm^2) by cells of plasma-derived fibrin hydrogels at 1.2 mg/ml and 2.4 mg/ml final fibrin concentration.

2.3.7 Fibroblast proliferation assay

Metabolic activity and proliferation of human primary fibroblast inside the two different plasma hydrogels was studied through Alamar BlueTM assay. The measurement is based on the fluorescence of the compound generated after metabolically active cells reduce resazurin into resorufin. This last component is highly fluorescent and can be measured in the micro plate reader with 530 nm excitation wavelength and 590 nm emission wavelength filters. Plasma hydrogels at the two studied concentrations of fibrin (1.2 mg/ml and 2.4 mg/ml) were prepared in black 96-well plates and incubated at 37°C and 5% CO₂ between each time point (1, 3, 7 and 14 days).

Results obtained evidence the different behaviour of fibroblasts inside the hydrogels systems. As expected from the results obtained in other previous experiments (structural characterisation and fibroblast-mediated contraction) fibroblast embedded in gels containing 2.4 mg/ml of final fibrin concentration showed a lagging behaviour in terms of cell proliferation (Figure 2.20). Metabolic activity of fibroblast incubated in 1.2 mg/ml plasma hydrogels was always higher than the one recorded for 2.4 mg/ml hydrogels. At day 14, the differences in proliferation for both systems remains. However hydrogels at 1.2 mg/ml and 2.4 mg/ml had similar proliferating rates taking into account the activity at day 7. This evidenced the ability of fibroblast to begin spreading inside the 2.4 mg/ml fibrin hydrogels after a no proliferation period of time due to a more concentrated matrix that impeded the cell division (Days 3 and 7).

2.3.8 Live/Dead assay

Further characterisation of the cytotoxicity of the plasma hydrogels was achieved through the Live/Dead[®] assay. This assay served as the proof that human primary fibroblasts can be embedded inside the plasma hydrogels systems and survive by spreading and dividing inside the gels. Plasma hydrogels containing 1.2 or 2.4 final fibrin concentrations and 80,000 cells/ml of human primary fibroblast were prepared in 8 μ -slide glass bottom Ibidi[®] plates and incubated for 48 hours with supplemented medium (10% FBS and 1% P/S) before taking them to the confocal microscope for their visualization.

Results show that fibroblast morphology is maintained regardless of the

Figure 2.20: Fibroblast proliferation inside the plasma hydrogels at 1.2 mg/ml and 2.4 mg/ml final fibrin concentration measured through Alamar BlueTM assay.

amount of final fibrin concentration used (1.2 mg/ml vs. 2.4 mg/ml), however, as previously reported by the Alamar BlueTM assay, proliferation was found to decrease for the 2.4 mg/ml fibrin concentration hydrogels (Figure 2.21).

Figure 2.21: Fibroblast proliferation inside the plasma hydrogels at 1.2 mg/ml and 2.4 mg/ml final fibrin concentration measured through Live/Dead[®] cytotoxicity assay after 48 hours incubation.

2.3.9 Keratinocytes proliferation assay (MTS)

Human primary keratinocytes ability to attach and proliferate on top of the hydrogels was studied through the MTS assay. Hydrogels at 1.2 mg/ml and 2.4 mg/ml of fibrin concentration were prepared on black 96-well plates in the absence of human fibroblast in order to avoid measuring cross-activity from this type of cells when recording the activity of primary keratinocytes. Keratinocytes were seeded on top at a density of 100,000 cells/ml and left untouched for 48 hours before starting the experiment. Hydrogels were maintained with special commercial medium CNT57 and incubated at 37°C and 5% CO₂ between recording times (0, 4 and 7 days).

The results obtained for the proliferation of keratinocytes on top of the hydrogels systems show that the initial activity of cells is very similar between both 1.2 mg/ml and 2.4 mg/ml hydrogels. However, after 4 and 7 days of culturing, keratinocytes proliferated at a stable rate for the 1.2 mg/ml fibrin hydrogels while their proliferation stagnated in the case of the more concentrated plasma hydrogels (Figure 2.22).

Figure 2.22: Keratinocytes proliferation at different time-points seeded on top of the plasma hydrogels at 1.2 mg/ml and 2.4 mg/ml final fibrin concentration measured through MTS assay.

2.3.10 Contraction in organotypic skin constructs (z-axis)

The contraction in the z-axis was evaluated during the process of skin maturation *in vitro* through photographs that were later analysed with ImageJ software. The results obtained show a decrease in the contraction for hydrogels containing 2.4 mg/ml of final fibrin concentration. This result was expected in terms of the overall decrease of proliferation found for fibroblast embedded on more concentrated plasma hydrogels. The differences found were of around 10-12% in height for the first 7 days, while after 10 days this difference increased to 14% and further increased to around 18% at time points 15 and 21 days (Figure 2.23).

Figure 2.23: Contraction ratio of organotypic skin constructs with final fibrin concentration of 1.2 mg/ml and 2.4 mg/ml.

2.4 Discussion

In this chapter, two different final concentrations of fibrin for the generation of dermo-epidermal equivalents have been studied, 1.2 mg/ml and 2.4 mg/ml. The rationale behind the performance of a series of experiments was to evaluate the suitability of the fibrin concentration to generate a matrix where cells can proliferate, migrate and remodel the structure. In order to do so, gelation time

and kinetics, contractile behaviour in the presence and absence of cells, internal structure, protein release, mechanical properties and human primary fibroblast and keratinocytes viability were evaluated.

Gelation time is a key aspect for the formation of hydrogels as it determines their capacity to encapsulate cells that occupy the entire matrix instead of sedimenting to the bottom of the hydrogel. It is also important regarding future applications of these hydrogels in terms of bioprinting extrusion seeking to automatize the process of generating TE skin [17, 18]. Gelation time and kinetics were studied through flip-flop and turbidity methods and a small difference of around two minutes was found between the 1.2 mg/ml and 2.4 mg/ml fibrin concentration hydrogels. It is well known that the turbidity increase is directly related with the cross-sectional area of the fibres forming the hydrogel [19]. Gelation or clotting point differed from the polymerization kinetics as expected since the time when the liquid stop flowing occurs quite rapid at the beginning of the polymerization process (15%-20%) as previously reported [19]. Furthermore the increase in gelation time measured through UV spectroscopy was found to be due to the higher amounts of fibrin present in the mixture, which delayed the complete polymerization of the material as previously reported [20].

Regarding the swelling behaviour of the two systems, the weight contraction when submerged in PBS at 37°C was measured. The ability of hydrogels to uptake and release great amounts of water is closely related with the presence and the balance of hydrophilic and hydrophobic groups in their structure [21]. No differences in terms of weight changes were recorded for the fibrin hydrogels at 1.2 mg/ml and 2.4 mg/ml of concentration. An overall 25% mass loss was found after the first 24 hours, with no further weight decrease for the next time points (up to 10 days). We hypothesize that a balance between the more compact matrix for 2.4 mg/ml hydrogels and the higher amount of proteins released for this condition are the reason behind the similar behaviour between the two concentration. More details about the contraction of fibrin hydrogels will be explained in section 3.4 of chapter 3. Parallely, the release of blood plasma proteins to the environment was measured yielding as result the expected double amount of proteins escaping the 2.4 mg/ml fibrin hydrogels as it contained double the amount of initial plasma. Interestingly the end of the release of the proteins present in the plasma hydrogels coincides with the end of the contraction for 1.2 mg/ml and 2.4 mg/ml hydrogels.

Mechanically, the hydrogels with different concentrations were found to have different performance under the uniaxial compression test. As expected, doubling the final concentration doubled the Young's modulus of the specimens in the compressive test, result similar to that reported on the effect of fibrin and thrombin concentration in the stiffness of the hydrogels [22]. Further characterisation of the polymeric matrices was assessed through the internal visualization of the hydrogels with SEM. Difference in the structural nature of the two concentrations evidenced the differences found in mechanical properties. Hydrogels at 1.2 mg/ml final fibrin concentration were found to be more porous and thus mechanically less stable.

Human primary fibroblast and keratinocytes were incorporated into the matrices to assess their biological properties in terms of contraction, proliferation and viability. Fibroblasts behaviour inside the matrices was correlated in terms of capability to contract the fibrin hydrogels and in terms of proliferation. The attachment of human primary fibroblast to the matrix through the RGD cell-adhesion motifs present in the matrix was confirmed as the hydrogels tend to contract at a bigger pace than in the absence of cells [23]. Cell-mediated contraction and proliferation were significantly decreased with hydrogels at 2.4 mg/ml with respect to the lower concentrated 1.2 mg/ml fibrin. This behaviour is in line with previous reports on the lower proliferation of cells with the increasing fibrin concentration due to a more compact matrix delaying the remodelling by the fibroblasts [22, 24]. Finally, human primary keratinocytes proliferation when seeded on top of the matrices showed a significant decrease in activity through the MTS assay. Contrary to what is found in the literature [25, 26], our result evidences that stiffer surfaces sensed by the cells are slowing the rate of proliferation for our system and thus are making the use softer matrices the best choice for the generation of the bioengineered skin. This effect might be due to not only the stiffness being responsible for the changes in proliferation but also the porosity and topography of the samples. The final characterisation of the cellular behaviour inside the matrices was the study of the contraction in the organotypic constructs. As the contraction was limited to the z-axis since the hydrogels were attached to the transwells walls, the height of the hydrogels was measured while the differentiation and maturation of the skin was taking place. Again, the results evidence that the lower proliferation of fibroblast inside the 2.4 mg/ml fibrin concentration is responsible for the lower degree of

contraction in the z-axis.

In this chapter 1.2 mg/ml hydrogels have shown worse mechanical properties and cell-mediated contraction rates, these negative results have been outweighed by their better or equal performance in terms of cell-free contraction, lower gelation times and most importantly human primary fibroblast and keratinocytes proliferation and viability, the key aspects for the correct wound healing of damaged skin [1]. In that sense, 1.2 mg/ml fibrin concentration hydrogels were selected to conduct a further matrix characterisation and to generate our dermo-epidermal equivalents seeking to enhance their performance through the incorporation of other biomaterial, the hyaluronic acid (HA).

Bibliography

- [1] S. Cox, M. Cole, and B. Tawil, "Behavior of Human Dermal Fibroblasts in Three-Dimensional Fibrin Clots: Dependence on Fibrinogen and Thrombin Concentration," *Tissue Engineering*, vol. 10, pp. 942–954, may 2004.
- [2] R. Asselta, S. Duga, and M. L. Tenchini, "The molecular basis of quantitative fibrinogen disorders," *Journal of Thrombosis and Haemostasis*, vol. 4, no. 10, pp. 2115–2129, 2006.
- [3] R. J. Faust, "Technical Manual of the American Association of Blood Banks," *Anesthesiology*, vol. 65, no. 2, p. 242, 1986.
- [4] A. Meana, J. Iglesias, M. Del Rio, F. Larcher, B. Madrigal, M. Fresno, C. Martin, F. San Roman, and F. Tevar, "Large surface of cultured human epithelium obtained on a dermal matrix based on live fibroblast-containing fibrin gels," *Burns*, vol. 24, no. 7, pp. 621–630, 1998.
- [5] J. L. Vanderhooft, M. Alcoutlabi, J. J. Magda, and G. D. Prestwich, "Rheological Properties of Cross-Linked Hyaluronan–Gelatin Hydrogels for Tissue Engineering," *Macromolecular bioscience*, vol. 9, no. 1, pp. 20–28, 2009.
- [6] B. Jeong, Y. H. Bae, and S. W. Kim, "Thermoreversible gelation of peg-plga- peg triblock copolymer aqueous solutions," *Macromolecules*, vol. 32, no. 21, pp. 7064–7069, 1999.
- [7] S. A. Smith, R. J. Travers, and J. H. Morrissey, "How it all starts: initiation of the clotting cascade," *Critical reviews in biochemistry and molecular biology*, vol. 50, no. 4, pp. 326–336, 2015.
- [8] M. M. Bradford, "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding," *Analytical Biochemistry*, vol. 72, pp. 248–254, may 1976.
- [9] T. P. Habif, *Clinical Dermatology E-Book: A Color Guide to Diagnosis and Therapy*. Elsevier Health Sciences, 2015.
- [10] "Instron 3360 Series - Instron. Retrieved from: <https://www.instron.us/products/testing-systems/universal-testing-systems/electromechanical/3300/3360-dual-column>. Access date: 2019-09-09."

- [11] “Carbon dioxide p-T phase diagram.svg - Wikimedia Commons. Retrieved from: https://commons.wikimedia.org/wiki/File:Carbon_dioxide_p-T_phase_diagram.svg. Access date: 2019-09-10.”
- [12] “Waters Prep Supercritical Fluid Extraction (SFE). Retrieved from: https://www.waters.com/waters/en_US/SFE-extraction-equipment/nav.htm?cid=134614431&locale=en_US. Access date: 2019-09-10.”
- [13] L. Reimer, *Scanning electron microscopy: physics of image formation and microanalysis*, vol. 45. Springer, 2013.
- [14] “Philips XL30 ESEM by SEMTech Solutions, Inc. Retrieved from: <https://www.used-line.com/microscopes/electron-microscopes/philips-xl30-esem/item-12499329>. Access date: 2019-09-10..”
- [15] “AlamarBlue HS and AlamarBlue Cell Viability Assay Protocol - ES. Retrieved from <https://www.thermofisher.com/>.”
- [16] Student, “The probable error of a mean,” *Biometrika*, pp. 1–25, 1908.
- [17] S. V. Murphy, A. Skardal, and A. Atala, “Evaluation of hydrogels for bio-printing applications,” *Journal of Biomedical Materials Research Part A*, vol. 101, no. 1, pp. 272–284, 2013.
- [18] N. Cubo, M. Garcia, J. F. Del Canizo, D. Velasco, and J. L. Jorcano, “3D bioprinting of functional human skin: production and in vivo analysis,” *Biofabrication*, vol. 9, p. 15006, dec 2016.
- [19] I. N. Chernysh and J. W. Weisel, “Dynamic imaging of fibrin network formation correlated with other measures of polymerization,” *Blood*, vol. 111, no. 10, pp. 4854–4861, 2008.
- [20] J. Wedgwood, A. J. Freemont, and N. Tirelli, “Rheological and turbidity study of fibrin hydrogels,” in *Macromolecular Symposia*, vol. 334, pp. 117–125, Wiley Online Library, 2013.
- [21] D. Velasco, C. Elvira, and J. San Román, “New stimuli-responsive polymers derived from morpholine and pyrrolidine,” *Journal of Materials Science: Materials in Medicine*, vol. 19, no. 4, pp. 1453–1458, 2008.

- [22] H. Duong, B. Wu, and B. Tawil, “Modulation of 3d fibrin matrix stiffness by intrinsic fibrinogen–thrombin compositions and by extrinsic cellular activity,” *Tissue Engineering Part A*, vol. 15, no. 7, pp. 1865–1876, 2009.
- [23] P. A. Janmey, J. P. Winer, and J. W. Weisel, “Fibrin gels and their clinical and bioengineering applications,” *Journal of the Royal Society Interface*, vol. 6, no. 30, pp. 1–10, 2008.
- [24] M. E. Smithmyer, L. A. Sawicki, and A. M. Kloxin, “Hydrogel scaffolds as in vitro models to study fibroblast activation in wound healing and disease,” *Biomaterials science*, vol. 2, no. 5, pp. 634–650, 2014.
- [25] Y. Wang, G. Wang, X. Luo, J. Qiu, and C. Tang, “Substrate stiffness regulates the proliferation, migration, and differentiation of epidermal cells.,” *Burns : journal of the International Society for Burn Injuries*, vol. 38, pp. 414–420, may 2012.
- [26] H. Zarkoob, S. Chinnathambi, J. C. Selby, and E. A. Sander, “Substrate deformations induce directed keratinocyte migration.,” *Journal of the Royal Society, Interface*, vol. 15, jun 2018.

Analysis of the contraction of plasma-derived fibrin matrices

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3.1 Introduction

Contraction of fibrin matrices is one of the key parameters hindering the standardization of these hydrogels as skin substitutes for drug testing and transplantation. Apart from the well known cell-mediated contraction of these matrices produced by the residing fibroblasts [1], other factors like fibrin matrix conformational changes modulated by the release of proteins also affect the behaviour of plasma hydrogels. Furthermore, low mechanical properties for fibrin-based matrices are also impeding their implementation as both testing platforms and skin substitutes due to their fast degradation *in vitro* [2].

The transportation of the dermo-epidermal equivalents from the site of generation to the clinic for transplantation is a key factor in the success of the skin transplants. Changes in the nature of the hydrogels during transportation is one of the persisting issues related with the plasma-derived hydrogels. Excessive contraction of the hydrogels led the researchers to incorporate a surgical glue to attach the skin sheets to a mesh that attenuates this effect. Far from ideal, this solution needs to be improved introducing changes in the structure that permit the inhibition of the contraction while being transported.

After the selection of a final fibrin concentration of 1.2 mg/ml to be the condition used for the dermo-epidermal equivalent from plasma-derived hydrogels, the effect of the temperature in the contractile behaviour of the matrix was studied. The characterisation of the behaviour of the matrix under different conditions allows us to better understand the processes occurring in the hydrogels and better design a strategy to reduce the detrimental behaviour of the equivalents.

3.2 Materials & Methods

3.2.1 Materials

Materials used in this chapter include the ones previously described in section 2.2.1 of Materials & Methods Chapter 2. Frozen human plasmas with low content of platelets were obtained from a blood bank in Spain (Banco de Sangre del Centro Comunitario de Transfusión del Principado de Asturias (CCST)) and treated according to the standards of the American Association of Blood

Banks [3]. Commercial fibrinogen from human plasma (Sigma Aldrich, USA) was used in conjunction to thrombin obtained as a raw material (Sigma Aldrich, USA). Each thrombin vial contains more than 10 National Institute of Health (NIH) units of thrombin [4]. Antifibrinolytic agent Amchafibrin (Tranexamic acid 500 mg, Meda Pharma SL, Spain), sodium chloride (NaCl 0.9%) and calcium chloride (CaCl_2 1%) (Sigma Aldrich, USA) were used for hydrogels formation. Phosphate-buffered saline (PBS) (Sigma Aldrich, USA) was used to incubate hydrogels. Bradford assay reagent (Bio-Rad Protein Assay Dye Reagent Concentrate) (Bio-Rad, Germany) and bovine serum albumin (BSA) (1 mg/ml) (Sigma Aldrich, USA) were used to measure protein release. Different dilutions of pure ethanol were used to dehydrate hydrogels for structural characterisation. Liquid carbon dioxide was purchased from Carburos Metálicos with a 99.99% purity. Strain-controlled AR-2000ex rheometer (TA Instruments) was used for rheological characterisation.

3.2.2 Plasma-derived fibrin hydrogels preparation

Human plasma-derived fibrin hydrogels were prepared as explained in section 2.2.2 of Materials & Methods Chapter 2. Human plasma aliquots of known fibrinogen concentration were defrosted and used in combination with antifibrinolytic agent Amchafibrin (0.008% w/v), and calcium chloride (CaCl_2) (0.08% w/v) plus sodium chloride (NaCl) to generate hydrogels with final concentration of 1.2 mg/ml of fibrin. Mixtures were left in the incubator at 37°C and 5% CO_2 for 1 hour in order for them to become a gel.

3.2.3 Commercial fibrinogen hydrogels preparation

Control hydrogels made of fibrinogen from human plasma (Sigma Aldrich, USA) were generated at a final fibrinogen concentration of 1.2 mg/ml. Commercial fibrinogen can be dissolved at a maximum of 10 mg/ml (1% w/v) in NaCl (0.9%) solution, thus calculations were first made to dissolve the fibrinogen and then generate the hydrogels. Fibrinogen was freshly prepared every time hydrogels were prepared and the product was stored at -20°C.

Contrary to the plasma hydrogels protocol, one component was missing in order to generate the commercial fibrinogen gel. Thrombin (Sigma Aldrich,

USA), the enzyme responsible for the polymerization of fibrinogen into insoluble fibrin, needed to be added to the mixture in order to synthesize the hydrogels. Thrombin was purchased as a raw material in vials containing 10 National Institute of Health (NIH) units, equivalent to 0.324 ± 0.073 μg of thrombin. In order to dissolve thrombin, 1 ml of NaCl 0.9% was added into the vials. The amount of thrombin needed to generate the gels was calculated knowing that human plasma contains between 5 and 10 mg/dL of the material [4, 5].

Commercial fibrinogen hydrogels were prepared in 19 mm diameter glass vials and contained NaCl 0.9%, CaCl_2 at a final concentration of 0.08% w/v, thrombin, antifibrinolytic Amchafibrin (0.08% w/v) and finally the human fibrinogen at a final concentration of 0.12% w/v. All materials were quickly mixed in order to avoid gelation and left in the incubator at 37°C 5% CO_2 for 1 hour for them to completely form a gel.

3.2.4 Gelation time and kinetics

Plasma and commercial fibrinogen hydrogels gelation time and kinetics were studied through both the flip-flop method and turbidimetry assay in order to determine the time and kinetics of the gelation process. Same procedure as explained in section 2.2.3 of Materials & Methods Chapter 2 was used to study the gelation. Hydrogels were prepared in 19 mm diameter glass vials and incubated at 37°C while the free flowing of the liquid was visually checked every thirty seconds. Gelation time was defined as the time when no free liquid is flowing inside the vials when these are inverted.

Furthermore, for the turbidimetry assay, first a UV scan from 200 nm to 800 nm wavelength with the liquid and gel forms of both plasma and commercial fibrinogen hydrogels was performed. This experiment allowed for the selection of the optimal wavelength at which the differences in O.D. from the solution and gel state were the maximum.

Finally, UV gelation was performed by measuring the O.D. dynamically every 30 seconds inside a 37°C pre-warmed SynergyTM HTX Multi-Mode Microplate Reader at the designated wavelength.

3.2.5 Hydrogel contraction at different temperatures

The effect of different temperatures (4°C, 23°C and 37°C) on the fibrin-based matrices was first studied in terms of mass swelling contraction. Plasma and commercial fibrinogen hydrogels were detached from the glass vials with the aid of 37°C pre-warmed PBS and placed into pre-weighted p60 culture dishes. Hydrogels were taken out of the vials following the procedure explained in section 2.2.4 of Materials & Methods Chapter 2. Initial weight (W_0) was calculated after detaching the hydrogels and placing them into the petri dish by measuring it in a precision scale. Hydrogels were then incubated at the different temperatures in 7 ml PBS and their weight differences measured at each time point (0h, 1h, 2h, 4h, 6h, 8h, 24h, 48h and 72h) by removing the PBS and carefully drying the plate with cotton swabs before putting them inside the precision scale. The swelling ratio was calculated using the following equation 3.1:

$$SR_W = \frac{W_T}{W_0} \quad (3.1)$$

After the measurements, fresh previously conditioned PBS was added to the plates and the incubation time count was resumed.

3.2.6 Fibrinogen hydrogel degradation assay

Commercial fibrinogen hydrogels degradation was assessed through a degradation assay in PBS at 37°C that consisted on the comparison in weight of the lyophilized hydrogels at different time-points. Three hydrogels per time-point were prepared as explained in section 3.2.3 with a final volume of 1.5 ml per gel. Samples were detached from the glass-vials and incubated in 7 ml of PBS at 37°C for time-points 0, 1, 7, 14 and 21 days. At each of these time-points, hydrogels were removed from the incubator and freeze dried in a LABCONCO freeze dryer. Dried hydrogels were weighted in p35 dishes and the measurement repeated for each hydrogel and time-point. Results were expressed as final dried weight in milligrams.

3.2.7 Protein release

The cumulative release of proteins from plasma and commercial fibrinogen hydrogels was studied as explained in section 2.2.5 of Materials & Methods Chapter 2 through the Bradford assay. Aliquots taken out from the surrounding PBS at different temperatures (4°C, 23°C and 37°C) and time points (0h, 1h, 2h, 4h, 6h, 8h, 24h, 48h and 72h) were diluted and mixed with the Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad, USA) and the change in absorbance in the presence of proteins measured at 595 nm in the Biowave II spectrophotometer (Biochrom, UK). Standard curve previously calculated served to extrapolate the concentration of proteins present in the surrounding medium (Figure 2.14). Dilutions were taken into account in order to calculate the concentration of proteins per millilitre and the cumulative release was calculated by adding the total amount of protein material released at each time point.

3.2.8 Structural characterisation (SEM)

Human plasma and commercial fibrinogen hydrogels were structurally characterised as reported in section 2.2.7 of Materials & Methods Chapter 2 through scanning electron microscopy. Hydrogels were prepared as previously explained in 19 mm diameter glass vials with a final volume of 3 ml. Incubation at 4°C, 23°C and 37°C for 48 hours was made in order to account the effect of the temperature on the internal structure of the hydrogels. Prior to supercritical CO₂ point drying, samples had to be dehydrated through a series of water-ethanol dilutions (20%, 40%, 60%, 80% and 100%). Drying of the samples was carried out at the supercritical CO₂ reactor at CSIC under the conditions described in table 3.1.

Time (min)	Temperature (°C)	Pressure (bar)
10	35	0-100
90	35	100
30	35	100-0

Table 3.1: Supercritical CO₂ drying conditions.

Plasma and commercial fibrinogen hydrogels were then sectioned and gold-sputtered before their visualization in the scanning electron microscope at Uni-

versidad Carlos III de Madrid. ImageJ software was used to calculate the fibre width of the plasma and commercial fibrinogen hydrogels.

3.2.9 Rheological characterisation

Rheological characterisation of the hydrogels was performed with a strain-controlled AR-2000ex Rheometer (TA Instruments) (Figure 3.1). The samples were placed between circular (12 mm diameter) parallel plates and a 1000 μm gap was left to avoid the slippage of the hydrogels. Three different time-points were selected, 0, 2 and 7 days after incubation at the three defined temperatures in this chapter (4°C, 23°C and 37°C). During the experiment the temperature was controlled through a Peltier device. Two main experiments were performed in shear deformation mode. First, the dynamic shear modulus was measured as a function of strain by a dynamic strain sweep with amplitudes ranging between 0.1% and 20% and at a constant frequency of 0.3 Hz. This measurement allowed us to determine the range of strain amplitudes where hydrogels showed a linear region of viscoelasticity. The second experiment measured the dynamic frequency sweep between 0.05 and 70 Hz at a fixed strain, previously selected within the hydrogel linear region with the objective of studying the dependence of the dynamic shear modulus and loss factor on the frequency. Rheology served to analyse the following parameters that describe the rheological properties of the material: storage modulus (G'), the loss modulus (G''), the complex modulus magnitude $|G^*|$, ($|G^*|^2 = (G')^2 + (G'')^2$), and the loss factor ($\tan \delta = (G'')/(G')$, where δ is the phase angle between the applied stimulus and the corresponding response as a function of strain amplitude or frequency.

3.2.10 Statistical analysis

Statistical analysis was performed using IBM[®] SPSS software. All the data averages and standard deviations were subjected to Student's t-test [7] in order to determine potential statistical difference significance in the results. Three levels of significance were assessed: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 3.1: TA Instruments Rheometer AR-2000ex [6].

3.3 Results

3.3.1 Gelation time and kinetics

Plasma-derived hydrogels and commercial fibrinogen hydrogels gelation time was studied using the flip-flop method. Vials were checked every 30 seconds and results obtained show a significant difference between the gelation time of plasma hydrogels (13.4 ± 2.4 minutes) and commercial fibrinogen hydrogels (3.4 ± 0.5 minutes) (Figure 3.2).

Characterization of the gelation process was further assessed through the study of the polymerization kinetics by UV spectroscopy. The change in O.D. for both the commercial fibrinogen and plasma hydrogels was dynamically measured and followed every 30 seconds to accurately determine the kinetics of the polymerization reaction. This is a widely used technique by other groups that have followed the polymerization of both commercial fibrinogen hydrogels [8, 9] and fibrinogen isolated from human plasma [10]. In order to determine the

Figure 3.2: Gelation times using the flip-flop method for commercial fibrinogen hydrogels and plasma hydrogels at 1.2 mg/ml final fibrin concentration.

wavelength at which this process can be followed for commercial fibrinogen and plasma hydrogels a UV scan of the solution and gel states of both systems was performed from 200 nm to 800 nm. Results of this UV scan show a clear difference between the liquid state and the hydrogels state in both systems (Figure 3.3). In the case of commercial fibrinogen, the difference between the O.D. of the solution and the hydrogel state reached 1 O.D. at 350 nm wavelength (Figure 3.3A). In the case of plasma hydrogel, the maximum difference between solution and hydrogels state was of just 0.5 O.D. at 325 nm wavelength (Figure 3.3B). These two wavelengths were then selected to make the UV gelation experiments at constant temperature of 37°C and measurements every 30 seconds. These wavelengths fall inside the ones previously reported in literature that range between 300 nm and 550 nm, with lower wavelength being unsuitable due to potential interference with proteins and DNA [11].

UV gelation experiments were performed in a SynergyTM HTX Multi-Mode Microplate Reader pre-warmed to 37°C. The components for both commercial fibrinogen hydrogels and plasma hydrogels were mixed and quickly transferred into the plate reader for the dynamic study. The commercial fibrinogen hydrogels showed a quick build up of O.D. reaching a plateau at around 1 O.D. after 11 minutes (Figure 3.4A). The results for the plasma hydrogels were very dif-

Figure 3.3: UV-Vis scans from 200 nm to 800 nm for A) Commercial fibrinogen solutions and hydrogels B) Plasma solutions and hydrogels.

ferent, showing a long initial lag phase in which no O.D. change was recorded; followed by a polymerization phase in which a 0.5 O.D change was recorded until minute 25, where the process reached a plateau (Figure 3.4B).

Figure 3.4: Gelation kinetics experiments measuring the optical density build up A) At 350 nm and 37°C for commercial fibrinogen hydrogels and B) At 325 nm and 37°C for human plasma hydrogels.

3.3.2 Hydrogels contraction at different temperatures

Human plasma and commercial fibrinogen hydrogels swelling behaviour was studied through the differences in weight after incubation in PBS at the three studied temperatures (4°C, 23°C and 37°C). Plasma hydrogels show a deswelling behaviour with initial differences between temperatures in terms of contraction, obtaining higher degree of mass loss at the extreme temperatures (4°C and 37°C) and a lower degree of contraction at 23°C for the first 8 hours of incu-

bation. After 24 hours of incubation at the different temperatures, all plasma hydrogels showed continuous mass loss at a slower rate, with final contraction of around 40% and no significant differences between temperatures (Figure 3.5).

Figure 3.5: Contraction of plasma hydrogels in terms of weight loss at the three different temperatures (4°C, 23°C and 37°C).

Commercial fibrinogen hydrogels showed bigger differences between temperatures in term of mass contraction. All fibrinogen hydrogels reached a stable mass loss state after 24 hours. Hydrogels exposed to 4°C were the condition that lost more mass, reaching a plateau after 24 hours of incubation at a final mass of just 25% of their initial weight. Physiological temperature (37°C) showed a mass loss of 55% of its initial weight. Finally, and following the result obtained for plasma hydrogels, 23°C was the condition that better reduced the contraction of the matrices. These hydrogels reached a plateau state at around a 30% of weight loss (Figure 3.6).

3.3.3 Fibrinogen hydrogel degradation assay

The degradation of commercial fibrinogen hydrogels was measured through the changes in weight of the lyophilized matrices at different time-points (1, 7, 14

Figure 3.6: Contraction of commercial fibrinogen hydrogels in terms of weight loss at the three different temperatures (4°C, 23°C and 37°C).

and 21 days). Results show that commercial fibrinogen hydrogels do not lose any weight after 21 days in PBS at 37°C (Figure 3.7). The initial weight of the hydrogels was slightly below 15mg, a weight that was maintained throughout the experiments with minor variations for days 1, 7, 14 and 21.

3.3.4 Protein release

Protein release from the plasma hydrogels was confirmed through the classical Bradford assay. Results show that plasma hydrogels release the proteins progressively with almost all plasma proteins out of the matrices after 48 hours of incubation for all temperatures (4°C, 23°C and 37°C). Differences in the amount of protein released among the temperatures were found from early time-points in the release study. After 8 hours, 4°C hydrogels had released the least amount of proteins at 11.2 mg, while at 23°C, hydrogels had release 15.7 mg of protein. Finally, 37°C was the condition that most protein released, with a total amount of almost 23 mg (Figure 3.8). In the case of commercial fibrinogen hydrogels and as expected, no release of proteins was found from their structure. These findings evidence the partial independence of mass loss from the protein release and reaffirm the effect of different temperatures on the fibrin matrix itself.

Figure 3.7: Dried weight changes recorded for commercial fibrinogen hydrogels incubated at 37°C in PBS at time-points 0, 1, 7, 14 and 21 days.

Figure 3.8: Cumulative protein release of plasma hydrogels at the three different temperatures (4°C, 23°C and 37°C).

Furthermore, a visual inspection of hydrogels was performed through photographs showing the progressive change in colour of the gel from a yellow tone

to a more white/pale tone (Figure 3.9). The presence of proteins in the human plasma is crucial for the correct wound healing when damage is produced. The main role played by this set of proteins is to enhance cell proliferation and migration, thus speeding the remodelling of skin and as a result decreasing the time for the tissue to repair [12].

Figure 3.9: Visual progress of hydrogels incubated at 4°C.

3.3.5 Structural characterisation (SEM)

Human plasma and commercial fibrinogen hydrogels were morphologically characterised through scanning electron microscopy (SEM). Hydrogels were first dried through the supercritical CO₂ point drying technique. The conditions studied were an initial plasma hydrogel; plasma hydrogel incubated for 48 hours at 4°C; human plasma hydrogel incubated at 37°C for 7 days and finally an initial commercial fibrinogen hydrogel as control.

The results obtained evidence the great differences in structural morphology of the hydrogels when they are incubated at different temperatures and times compared to the initial plasma and initial commercial fibrinogen hydrogels (Figure 3.10A and 3.10D). After incubation for 7 days at 37°C, fibres showed a smoother shape, changing their appearance to look more like the commercial fibrinogen fibres (Figure 3.10C). On the other hand, the incubation of the hydrogels at 4°C for 48 hours translated into a change in morphology of the hydrogels, with thicker fibres and a more compact aspect compared to the plasma and fibrinogen controls (Figure 3.10B).

Fibre diameter was calculated using the imaging software ImageJ and results show the difference that exists between the newly formed fibres and the commercial fibrinogen and plasma fibres after 7 days incubation at 37°C. Fi-

Figure 3.10: Scanning electron microscopy images of A) Plasma hydrogel at 0h; B) Plasma hydrogel 48h at 4°C; C) Plasma hydrogel 7 days at 37°C; D) Commercial fibrinogen hydrogel at 0h.

bre width for the hydrogel incubated at 4°C for 48 hours was maintained with respect to the initial plasma hydrogel, with an increase in the range of fibre diameters (Figure 3.11).

3.3.6 Rheological characterisation

Rheological experiments served the purpose to understand the behaviour of the hydrogels at different temperatures (4°C, 23°C and 37°C) in terms of shear forces and general viscoelastic behaviour. As explained in Materials & Methods section 3.2.9, first, a variable strain sweep study at a constant 0.3 Hz was performed to all hydrogels in order to determine the linear region of viscoelasticity, defined as the region where the storage (G') and loss modulus (G'') are parallel and independent of the strain. The results depicted in figure 3.12 show a central linear and parallel region where G' is always greater in value than G'' up to a point defined as critical deformation (γ_0) where the slope of the curve dramati-

Figure 3.11: Fibre diameters measured through ImageJ software.

cally changes at higher strain amplitudes, generating a non-linear region for G' and G'' . This region and the cross-point between these two magnitudes evidence the transition from a solid behaviour of the hydrogels to a liquid-like behaviour.

Figure 3.12: Strain sweep experiment at constant frequency 0.3Hz after 48 hours for plasma hydrogels at the three different temperatures (4°C, 23°C and 37°C).

Furthermore, loss modulus (G'') evolves with the change in strain by generating a maximum value before decreasing again at certain strain value (%).

This is known as the weak-strain hardening behaviour and is characteristic of the energy dissipation that occurs when the hydrogel breaks its structure.

The next step in the rheological characterisation was the study of the effect of frequency on the thermally treated hydrogels at a constant strain previously selected from the linear region of the strain sweep experiment. The selected strain was 1% and results for the hydrogels incubated at the respective temperatures for 48 hours show that all hydrogels present the same gel-like behaviour with two main characteristics. First, for all temperatures, hydrogels present a higher value for the storage modulus than the values of the loss modulus ($G' > G''$) and secondly, these values of elastic and viscous modulus respectively are independent of the frequency at the range tested (0.05 to 70 Hz) (Figure 3.13).

Figure 3.13: Frequency sweep experiment at constant strain 1% for plasma hydrogels kept for 48 hours at the three different temperatures (4°C, 23°C and 37°C).

The elastic modulus was used to compare the performance of the hydrogels after being subjected to the three different temperatures for different times (48 hours and 7 days) of incubation. The following figure 3.14 shows the differences in terms of elasticity of the hydrogels after thermal treatment compared to their initial performance. Hydrogels subjected to 48 hours of incubation at different temperatures showed an increase in elastic modulus for the 4°C and 23°C conditions, while the hydrogels at 37°C maintained modulus values simi-

lar to the ones recorded initially. On the other hand, after 7 days of incubation, elastic modulus decreased for all temperatures with respect to the initial value except for the 4°C hydrogels that maintained the mechanical performance (Figure 3.14).

Figure 3.14: Elastic modulus of plasma hydrogels at the three different temperatures (4°C, 23°C and 37°C) and two different time-points (48 hours and 7 days).

3.4 Discussion

The effect of temperature into the plasma-derived and commercial fibrinogen matrices was studied thoroughly in this chapter. This study gave us the chance to further characterise one of the processes that occur to the hydrogels after they are formed and need to be transported into the clinic for transplantation purposes. Apart from the range of temperatures in which this transportation might occur, extreme temperature and physiological temperature were used seeking to better understand the changes occurring internally and opening the possibility for intact conservation of the matrices to get an off the shelf supply for patients.

Three different temperatures were used (4°C, 23°C and 37°C) in this study that comprised a series of experiments devoted to the characterisation of the matrices applying material science techniques.

Regarding gelation time, commercial fibrinogen hydrogels gelled almost im-

mediately after mixture and showed a fragile aspect due to the lack of components providing additional mechanical support the structure and to its very low final fibrin concentration of 1.2 mg/ml (Figure 3.2). In the literature these hydrogels are usually generated at concentrations ranging between 10 and 200 mg/ml [13]. On the contrary, plasma hydrogels contain proteins and lipids that are trapped during gelation providing mechanical stability to the hydrogels and making the gelation time to be delayed due to steric effects [14].

The differences in final O.D. between the commercial fibrinogen hydrogels and the plasma hydrogels result from the initial turbidity of the liquid state plasma is already of around 0.5 O.D. due to the presence of proteins and lipids. Commercial fibrinogen solutions, on the other hand, are initially completely transparent showing no absorbance and they quickly change into opaque hydrogels showing a final value of 1 O.D.

Comparing both gelation methods, flip-flop gives information about when visually the hydrogels had formed, while the UV gives a deeper insight into the polymerization reaction of fibrinogen taking place. Thus, while externally the hydrogels were formed and no liquid could flow inside the vials, internally the reaction continued occurring until the O.D. reached a plateau. The gelation time and kinetics study shows that the proteins and lipids contained in human plasma are capable of delaying the polymerization reaction.

The results obtained for the contraction of both human plasma and commercial fibrinogen hydrogels show that all matrices contract after incubation in an aqueous environment regardless of the temperature (Figures 3.5 and 3.6). Plasma hydrogels show a bi-phasic deswelling behaviour with an initial quick loss of mass up to 24 hours. This contraction is explained by the intrinsic nature of the fibrin hydrogels to deswell in aqueous solution as shown in figure 3.6. However, the lower loss of mass reported for plasma hydrogels is due to the presence of proteins and lipids in their structure, that are capable of modulating the contraction. The second phase of contraction starts after 24 hours and coincides with the end of the release of almost all plasma proteins detected by the protein release assay (Figure 3.8). However, a slower and less pronounced loss of mass is reported for plasma hydrogels, behaviour that could be explained by the presence of lipids in the structure and bigger proteins that have not been released yet. The huge change experienced by the hydrogels incubated at 4°C

is explained by the lower release of proteins found for this condition. Instead of losing 70% of their mass after 24 hours when produced with commercial fibrinogen, plasma hydrogels subjected to the same temperature were able to contract only 30% after the same amount of time. The presence of proteins and the bigger rate of retention at this temperature provoked the dramatic change in contractile behaviour found.

In this sense, contractile behaviour can be explained due to the release of proteins and internal changes in the fibrin matrix from the plasma hydrogels. The contraction of the commercial fibrinogen hydrogels in the same and even bigger manner suggest the effect of the temperature in the internal structure of the fibrin matrix is the main driver for contraction. Both the release of proteins and the bi-phasic behaviour of hydrogels previously reported by Zhang *et al.* for collagen hydrogels [11], showing an outer hydrophilic part and an inner hydrophobic part, could be the explanation for the contraction of the matrices. Regarding the contraction attributed to the matrix, Zhao *et al.* [10] reported a weight loss of 80% after 3 days incubation in PBS at 37°C for commercial fibrinogen hydrogels at different final concentrations. The authors explained the behaviour as fibrinogen dissolution due to the ionic strength of PBS causing the weight loss. However, attending to our results we do not appreciate any dissolution of the fibrinogen hydrogels with time since we did not observe any absorbance signal in the Bradford assay neither degradation in the fibrinogen hydrogels samples when they are lyophilized and weighted at different time-points (Figure 3.7). The explanation for the contraction thus lays in the two-phase behaviour previously reported as the main characteristic of environmental sensitive hydrogels containing both hydrophilic and hydrophobic chains in their structure [15, 16].

The contraction of fibrin matrices might be related with the aggregation of intermolecular assemblies when the hydrogels are submerged in aqueous solution. In this environment the non-polar groups attract to each other forming new crosslinking points that make the scaffold to be less porous and thus release more water to the environment, causing a mass decrease [11].

Structurally, differences were found between the initial plasma hydrogel and the gel incubated at 4°C for 48 hours. Aggregation of fibres seemed to occur, in line with the increase in O.D. found for commercial fibrinogen hydrogels in the

turbidimetry assay (Figure 3.4A). This effect has already been proven in other studies, suggesting that higher optical densities are an indicative of thicker fibres [17, 18]. Furthermore, no differences in fibre thickness nor porous structure was found between the plasma hydrogels after 7 days at 37°C and the initial commercial fibrinogen hydrogels.

The rheological study performed at the three defined temperatures showed the absence of what is known as the strain hardening effect. This effect is defined as the increase in elastic modulus (G') with the increasing values of strain amplitude and has been well characterised in commercial fibrinogen hydrogels [19, 20]. The presence of this effect in commercial fibrinogen structures is due to the flexible nature of the fibres in line with the polymer network theory of flexible chains that define the hardening of the fibres upon the application of increasing strains to the polymer network [21, 22]. In the case of plasma hydrogels, this effect was not found as evidenced in the plateau found in elastic modulus with the increasing strain amplitude when performing the strain sweep experiment (Figure 3.12). As previously reported by Shah *et al.* the elimination of this effect in plasma-derived hydrogels might be due to the presence of proteins and platelets in the structure [20].

Furthermore, the elastic modulus observed at 1% strain and 0.3 Hz of frequency for the hydrogels at different temperatures showed a decrease in modulus for the physiological temperature compared to the initial control. However, plasma hydrogels at 4°C after 7 days incubation maintained the mechanical properties probably due to the thickening of the fibres and the lower release of proteins reinforcing the matrices.

In this chapter we have thoroughly studied the fibrin matrix and its behaviour under different environments in terms of temperature. This is an important characterisation of the material before continuing to add a supplementary biomaterial (hyaluronic acid) in order to reinforce the matrix and enhance the biological properties.

Bibliography

- [1] Y. Chen, X. Shi-wen, J. van Beek, L. Kennedy, M. McLeod, E. A. Renzoni, G. Bou-Gharios, S. Wilcox-Adelman, P. F. Goetinck, M. Eastwood, *et al.*, “Matrix contraction by dermal fibroblasts requires transforming growth factor- β /activin-linked kinase 5, heparan sulfate-containing proteoglycans, and mek/erk: insights into pathological scarring in chronic fibrotic disease,” *The American journal of pathology*, vol. 167, no. 6, pp. 1699–1711, 2005.
- [2] B. Ruszymah, K. Chua, A. Mazlyzam, and B. Aminuddin, “Formation of tissue engineered composite construct of cartilage and skin using high density polyethylene as inner scaffold in the shape of human helix,” *International journal of pediatric otorhinolaryngology*, vol. 75, no. 6, pp. 805–810, 2011.
- [3] R. J. Faust, “Technical Manual of the American Association of Blood Banks,” *Anesthesiology*, vol. 65, no. 2, p. 242, 1986.
- [4] P. J. Gaffney and T. A. Edgell, “The International and "NIH" units for thrombin—how do they compare?,” *Thrombosis and haemostasis*, vol. 74, pp. 900–903, sep 1995.
- [5] E. G. Fischer, W. Ruf, and B. M. Mueller, “Tissue factor-initiated thrombin generation activates the signaling thrombin receptor on malignant melanoma cells.,” *Cancer research*, vol. 55, pp. 1629–1632, apr 1995.
- [6] “Rheometer – AR-2000EX | Dr. Yael Vodovotz. Retrieved from: <http://u.osu.edu/yaelvodovotz/instrumentation/rheometer-ar-2000ex/>. Access date: 2019-06-07.”
- [7] Student, “The probable error of a mean,” *Biometrika*, pp. 1–25, 1908.
- [8] K. M. Weigandt, N. White, D. Chung, E. Ellingson, Y. Wang, X. Fu, and D. C. Pozzo, “Fibrin clot structure and mechanics associated with specific oxidation of methionine residues in fibrinogen,” *Biophysical journal*, vol. 103, pp. 2399–2407, dec 2012.
- [9] J. Wedgwood, A. J. Freemont, and N. Tirelli, “Rheological and turbidity study of fibrin hydrogels,” in *Macromolecular Symposia*, vol. 334, pp. 117–125, Wiley Online Library, 2013.

- [10] H. Zhao, L. Ma, J. Zhou, Z. Mao, C. Gao, and J. Shen, “Fabrication and physical and biological properties of fibrin gel derived from human plasma.,” *Biomedical materials (Bristol, England)*, vol. 3, p. 15001, mar 2008.
- [11] X. Zhang, L. Xu, S. Wei, M. Zhai, and J. Li, “Stimuli responsive deswelling of radiation synthesized collagen hydrogel in simulated physiological environment.,” *Journal of biomedical materials research. Part A*, vol. 101, pp. 2191–2201, aug 2013.
- [12] R. Corrales, M. Sikorski, M. García, D. Velasco, and J. Jorcano, “Mechanical modulation of a human plasma based skin scaffold via reactive multi-arm polyethylene glycols,” *Biomecánica*, jul 2018.
- [13] M. P. Linnes, B. D. Ratner, and C. M. Giachelli, “A fibrinogen-based precision microporous scaffold for tissue engineering,” *Biomaterials*, vol. 28, pp. 5298–5306, dec 2007.
- [14] P. Arnaud, “The plasma proteins: Structure, function, and genetic control,” *Clinical Immunology and Immunopathology*, vol. 39, no. 3, p. 531, 2004.
- [15] J.-Y. Fang, J.-P. Chen, Y.-L. Leu, and J.-W. Hu, “Temperature-sensitive hydrogels composed of chitosan and hyaluronic acid as injectable carriers for drug delivery.,” *European journal of pharmaceuticals and biopharmaceutics : official journal of Arbeitsgemeinschaft fur Pharmazeutische Verfahrenstechnik e.V*, vol. 68, pp. 626–636, mar 2008.
- [16] A. Montero, L. Valencia, R. Corrales, J. L. Jorcano, and D. Velasco, “Smart Polymer Gels: Properties, Synthesis, and Applications,” *Smart Polymers and their Applications*, pp. 279–321, jan 2019.
- [17] V. Nehls and R. Herrmann, “The configuration of fibrin clots determines capillary morphogenesis and endothelial cell migration,” *Microvascular research*, vol. 51, no. 3, pp. 347–364, 1996.
- [18] W. Hayen, M. Goebeler, S. Kumar, R. Rießen, and V. Nehls, “Hyaluronan stimulates tumor cell migration by modulating the fibrin fiber architecture,” *Journal of cell science*, vol. 112, no. 13, pp. 2241–2251, 1999.

- [19] C. Storm, J. J. Pastore, F. C. MacKintosh, T. C. Lubensky, and P. A. Janmey, “Nonlinear elasticity in biological gels,” *Nature*, vol. 435, no. 7039, p. 191, 2005.
- [20] J. V. Shah and P. A. Janmey, “Strain hardening of fibrin gels and plasma clots,” *Rheologica Acta*, vol. 36, no. 3, pp. 262–268, 1997.
- [21] R. I. Litvinov and J. W. Weisel, “Fibrin mechanical properties and their structural origins.,” *Matrix biology : journal of the International Society for Matrix Biology*, vol. 60-61, pp. 110–123, jul 2017.
- [22] W. Li, J. Sigley, M. Pieters, C. C. Helms, C. Nagaswami, J. W. Weisel, and M. Guthold, “Fibrin fiber stiffness is strongly affected by fiber diameter, but not by fibrinogen glycation,” *Biophysical journal*, vol. 110, no. 6, pp. 1400–1410, 2016.

Incorporation of natural hyaluronic acid in plasma-derived fibrin hydrogels

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4.1 Introduction

The persisting issues associated with the human plasma-derived fibrin hydrogels for skin tissue engineering led us to come up with potential solutions that conferred better mechanical properties and inhibited contraction while being still clinically relevant. In that sense, the incorporation of a secondary material already present in the natural ECM and with a defined role in wound healing was proposed as the potential solution. Combination of several materials in order to reinforce matrices has already being used by different groups around the world [1, 2].

The combination of properties of two different materials can indeed change the behaviour of the hydrogels both *in vitro* and *in vivo*. Glycosaminoglycans (GAGs) have been the primary option to be incorporated into more traditional collagen and fibrin matrices to both improve the mechanical properties and also enhance the biological response in order to obtain better outcomes in wound healing. Among those GAGs, components naturally present in the skin dermal compartment and playing an important role in wound healing, hyaluronic acid (HA) have risen as a promising candidate due to its good compatibility, great water content and mechanical properties and its role in the foetal scarless wound healing [3–8].

In this chapter, unmodified, natural HA of three different molecular weights (MW) (500 kDa, 700 kDa and 1 MDa) and purchased from Lifecore[®] Biomedical [9] was used at physiological concentrations (0.05%, 0.1% and 0.2%) in order to try to improve the plasma matrices at 1.2 mg/ml fibrin concentration and overcome the cited persisting issues associated with them for their use in transplantation and *in vitro* skin models.

4.2 Materials & Methods

4.2.1 Materials

Materials used in this chapter include the ones previously described in section 2.2.1 of Materials & Methods Chapter 2. Frozen human plasmas with low content of platelets were obtained from a blood bank in Spain (Banco de Sangre del Centro Comunitario de Transfusión del Principado de Asturias (CCST))

and treated according to the standards of the American Association of Blood Banks [10]. Lifecore[®] Sodium Hyaluronate (MW 500 kDa, 700 kDa and 1 MDa) was obtained from Lifecore[®] Biomedical, USA [9]. Antifibrinolytic agent Amchafibrin (Tranexamic acid 500mg, Meda Pharma SL, Spain), sodium chloride (NaCl 0.9%) and calcium chloride (CaCl₂ 1%) (Sigma Aldrich, USA) were used for hydrogels formation. Phosphate-buffered saline (PBS) (Sigma Aldrich, USA) was used to incubate hydrogels. Bradford assay reagent (Bio-Rad Protein Assay Dye Reagent Concentrate) (Bio-Rad, Germany) and bovine serum albumin (BSA) (1 mg/ml) (Sigma Aldrich, USA) were used to measure protein release. Different dilutions of pure ethanol were used to dehydrate hydrogels for structural characterisation. As cell culture medium, Dulbecco's Modified Eagle's Medium (DMEM) + GlutaMAX[™] supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin (P/S) (Thermo Fisher Scientific, USA) were used. Cellular proliferation and viability was measured with Alamar Blue[™] Cell Viability Reagent.

4.2.2 Plasma derived hydrogels containing natural HA preparation

Human plasma-derived fibrin hydrogels were prepared as previously described in this thesis in section 2.2.2 Materials & Methods Chapter 2. For this set of experiments, hyaluronic acid of natural origin was purchased from Lifecore[®] Biomedical, USA [9]. Three different molecular weights were tested: 500 kDa, 700 kDa and 1 MDa. Hyaluronic acid was dissolved at a concentration of 10 mg/ml in NaCl 0.9% prior to use and agitated with magnetic bead and heat for half an hour until it completely dissolved.

Incorporation of the sodium hyaluronate into the plasma hydrogels protocol was performed as follows: the final fibrinogen concentration was maintained at 1.2 mg/ml, as well as the concentration of tranexamic acid, the antifibrinolytic agent at 0.008% w/v. Hyaluronic acid was then added at three final concentrations of 0.05%, 0.1% and 0.2% w/v. The volume needed to incorporate HA into the hydrogels was subtracted from the NaCl 0.9% that was normally added. Finally, CaCl₂ 1% at a concentration of 0.08% w/v was added in order to start the coagulation cascade and generate the hydrogels. The mixture was then put in the incubator at 37°C and 5% CO₂ for 1 hour to allow for the complete gelation.

4.2.3 Gelation time and kinetics

Hydrogels gelation time and kinetics were studied as previously reported in section 2.2.3 of Materials & Methods Chapter 2. Gelation time was studied through the classical flip-flop method as previously reported by Vanderhooft *et al.* and Jeong *et al.* [11, 12] and the time at which no liquid was free flowing inside the glass-vials was annotated as the gelation time. Plasma hydrogels containing natural HA were prepared in 19 mm diameter vials and were visually checked every 30 seconds to assess the gelation time.

Furthermore, gelation kinetics were studied through a turbidimetry assay in which the change of O.D. as the mixtures become hydrogels was recorded. In order to do so, first an UV scan from 200 nm to 800 nm was performed with the incorporated HA into the hydrogels to check if this change in the composition would change the O.D. spectrum. Later, the wavelength at which a maximum difference was found between the liquid and fully formed hydrogel samples was chosen to perform the UV gelation kinetics test. Gelation kinetics were recorded at 315 nm wavelength every 30 seconds in a SynergyTM HTX Multi-Mode Microplate Reader.

4.2.4 Hydrogels contraction in the presence of natural HA

The effect of the incorporation of natural HA into the plasma-derived fibrin matrices was assessed through a mass contraction study. Following the procedure explained in section 2.2.4 of Materials & Methods Chapter 2, hydrogels containing three types of HA (500 kDa, 700 kDa and 1 MDa) and three final concentrations (0.05%, 0.1% and 0.2%) were detached from the glass-vials and transferred to previously weighted p35 dishes. Initial weight (W_0) was calculated in a precision scale at the moment where the hydrogels were changed from the vial to the p35 just after gelation. Three millilitres of PBS were used to cover the samples during the experiment time-points (0h, 1h, 2h, 4h, 6h, 8h, 24h, 48h and 72h). At each of these time points, PBS was removed from the dishes and hydrogels dried and weighted in a precision scale. Swelling ratio was calculated using equation 4.1:

$$SR_W = \frac{W_T}{W_0} \quad (4.1)$$

The procedure was repeated for the remaining of the time points and fresh PBS was added after every measurement.

4.2.5 Protein release

The release of proteins naturally contained in plasma in the presence of natural HA was studied through the Bradford assay as explained in section 2.2.5 of Material & Methods Chapter 2. HA was incorporated into the plasma hydrogels as previously explained and the gels were incubated in PBS for 72 hours to check the release profile of the proteins and the effect of HA in the cumulative release. The time-points studied were the following: 0h, 1h, 2h, 4h, 6h, 8h, 24h, 48h and 72h. In order to measure the proteins that escape the hydrogels systems, 1 ml aliquots of the surrounding PBS were taken at each time point, diluted and mixed with the Bio-Rad protein assay reagent. This mixture was measured to check the change in absorbance at 595 nm in a Biowave II spectrophotometer. The dilution performed was taken into account to compute the amount of proteins released at each time point with a previously obtained standard curve (Figure 2.14) and the cumulative amount released was calculated by adding all the values. After every time point, PBS was replaced completely with fresh medium.

4.2.6 Viscosity of natural HA

Viscosity of the three sodium hyaluronates (500 kDa, 700 kDa and 1 MDa) was measured using a rotary viscometer (ST-2020) (Figure 4.1). A final volume of 5 ml of hyaluronic acid was prepared by dissolving 50 mg of HA powder in NaCl 0.9%. The mixture was left agitating with heat for half an hour before being tested for viscosity. The viscometer is capable of measuring viscosities in the range of 20 to 2,000,000 mPa · s (cP) by measuring the resistance exerted by the solution against a cylindrical rotating spindle. The viscometer probe was submerged into the dissolved HA and several readings were performed at 30 rpm for MWs 500 kDa and 700 kDa and of 0.6 rpm for the more viscous 1 MDa HA.

Figure 4.1: Viscosimeter used to characterize the viscosity of the three different natural HA used (500 kDa, 700 kDa and 1 MDa) [13].

4.2.7 Mechanical characterisation: compression test

The mechanical properties of plasma-derived hydrogels with incorporated natural hyaluronic acid were studied through a compression test carried out with an Instron 3366 machine. Following the protocol of compression explained in section 2.2.6 of Materials & Methods Chapter 2 the effect of the three variants of HA (500 kDa, 700 kDa and 1 MDa) at three different final concentrations (0.05%, 0.1% and 0.2%) were studied. In short, hydrogels containing natural HA were prepared at a final volume of 5 ml in 25 mm diameter glass-vials. Hydrogels were detached after 1 hour of gelation at 37°C and transferred to p60 culture dishes with 7 ml of PBS. Plasma hydrogels containing natural HA were left equilibrating for 24 hours before taking them to the facility where the mechanical test was performed.

Uniaxial compression test was carried out on the samples giving as data the load in Newtons (N) and the strain in percentage (%). This data in conjunction with the known diameter and height of the samples was used to calculate

the engineering stress (σ), engineering strain (ϵ) of the samples tested with the following equations 4.2 and 4.3:

$$\sigma = \frac{F}{A_0} \quad (4.2)$$

$$\epsilon = \frac{\Delta L}{L_0} \quad (4.3)$$

Young's modulus or compressive modulus of elasticity (E) was calculated from these data at the linear region of 20% strain using the following equation 4.4:

$$E = \frac{\sigma(\epsilon)}{\epsilon} = \frac{F/A}{\Delta L/L_0} \quad (4.4)$$

4.2.8 Cell culture

As explained in section 2.2.8 of Materials & Methods Chapter 2, human primary fibroblast obtained from skin biopsies were used to perform the cellular experiments involving the encapsulation of cells inside the plasma-derived fibrin matrices. Fibroblasts were stored in liquid nitrogen at -195.8°C and defrost and seeded into T75 flask before the start of each experiment. Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin/Streptomycin (P/S) antibiotic/antimycotic agent was used as culture medium. Cells were maintained at 37°C and 5% CO_2 pressure in the incubator and medium was changed every 2 days until fibroblast reached confluence.

Detachment from the T75 flask proceeded as follows: pre-warmed PBS was added into the culture flask after medium was vacuumed in order to remove any excess of supplemented DMEM. Two millilitres of trypsin were added into the flask and left incubation for 10 minutes at 37°C in order to detach the cells from the bottom. The flask was visualised under the microscope to assure all

the fibroblast were floating and trypsin was inactivated by adding supplemented medium on top. Fibroblast were then counted by putting 10 μ l of the solution into a Neubauer chamber and counting under the microscope in order to assess the number of cells. The solution containing the fibroblast was then centrifuged for 7 minutes and 1200 rpm after which the supernatant was discarded and the pellet of cells was re-suspended in the desired amount of supplemented medium to perform the experiment.

4.2.9 Fibroblast-mediated matrix contraction

The capacity of natural HA to inhibit the contraction of hydrogels in the presence of human primary fibroblast was studied through an area contraction assay. As reported in section 2.2.9 of Materials & Methods Chapter 2, hydrogels containing natural HA were modified to also incorporate primary fibroblast at a density of 80,000 cells/ml. Three levels of HA concentration (0.05%, 0.1% and 0.2%) and three molecular weights (500 kDa, 700 kDa and 1 MDa) were studied at the following time points: 0h, 4h, 6h, 8h, 24h, 48h, 72h, 7 days, 10 days and 15 days.

Photographs of the hydrogels incubated in DMEM with a 10% FBS and 1% P/S taken at each time point were analysed to calculate the area with imaging software ImageJ. Swelling area was calculated using the following equation 4.5:

$$SR_A = \frac{A_T}{A_0} \quad (4.5)$$

4.2.10 Fibroblast proliferation assay

Potential toxic effect of the incorporated natural HA was assessed as previously explained in section 2.2.10 of Materials & Methods Chapter 2 through a commercial proliferation assay (Alamar BlueTM). Plasma hydrogels containing natural HA were prepared in black 96-well plates at a final volume of 100 μ l per gel and in the presence of human primary fibroblast at a density of 80,000 cells/ml of gel. Samples were incubated in supplemented medium between time

points (1 day, 3 days, 7 days and 14 days) before taken out of the incubator for their measurement. Alamar BlueTM cell viability reagent was used to measure the cellular activity by incubating the diluted reagent (10%) with the hydrogels for 3 hours at 37°C and 5% CO₂. Changes in fluorescence in the presence of metabolically active fibroblast were measured in the SynergyTM HTX Multi-Mode Microplate Reader at 570 nm excitation and 600 nm emission wavelengths.

4.2.11 Statistical analysis

Statistical analysis was performed using IBM[®] SPSS software. All the data averages and standard deviations were subjected to Student's t-test [14] in order to determine potential statistical difference significance in the results. Three levels of significance were assessed: *p<0.05; ** p<0.01; *** p<0.001.

4.3 Results

4.3.1 Gelation time and kinetics

The effect of the incorporation of natural HA into the plasma matrices on the gelation time was studied through the flip-flop method. The results obtained show that adding diverse amounts (0.05%, 0.1% and 0.2%) and molecular weights (500 kDa, 700 kDa and 1 MDa) of HA indeed affects the gelation times by delaying the completion of the process.

Lower molecular weight, 500 kDa, was the condition that did not affect the gelation time with independence of the amount of HA incorporated to the matrices (Figure 4.2A). On the other hand, incorporation of both 700 kDa and 1 MDa HA did significantly affect gelation times by delaying the process progressively as the amount of HA is increased (Figures 4.2B and 4.2C). Hydrogels gelation was delayed from the 12.75 ± 0.5 minutes of the plasma hydrogels to the more than 22 minutes in case of HA 0.2% 700 kDa and up to 35.6 ± 1.5 minutes for the HA 0.2% 1 MDa hydrogels. The same data represented in an alternative manner in figure 4.3 show the effect of HA MW on the gelation time.

Gelation kinetics of hydrogels with incorporated natural HA were studied

Figure 4.2: Gelation times with the flip-flop method for hydrogels containing three concentrations of HA (0.05%, 0.1% and 0.2%) for each MW A) 500 kDa, B) 700 kDa and C) 1 MDa.

Figure 4.3: Gelation times with the flip-flop method for hydrogels containing three levels of MW (500 kDa, 700 kDa and 1 MDa) for each HA concentration A) 0.05%, B) 0.1% and C) 0.2%.

through UV-Vis spectroscopy. First, UV scans were performed to determine if the incorporation of HA affected the O.D. spectrum. It was found that HA did not affect the O.D. and thus the wavelength (315 nm) at with the difference in O.D. between the liquid and gel state was the highest was selected to perform the UV gelation experiments (Figure 4.4A, 4.4B and 4.4C).

UV gelation experiments were performed at the cited wavelength (315 nm), yielding as result an homogeneous behaviour in terms of polymerization measured through turbidity build up for all the samples (Figure 4.5A, 4.5B and 4.5C). Difference in optical density from the solution to the hydrogels state was maintained for all hydrogels compositions HA (0.05%, 0.1% and 0.2%) and molecular weight levels (500 kDa, 700 kDa and 1 MDa).

4.3.2 Hydrogels contraction in the presence of natural HA

Human plasma hydrogels containing natural HA were studied in a mass contraction experiment in order to determine the effect of HA on the contraction of the hydrogels. All three levels of HA concentration (0.05%, 0.1% and 0.2%) and molecular weights (500 kDa, 700 kDa and 1 MDa) were studied for 72 hours submerged in PBS at 37°C.

The results obtained show an increase in contraction when the amount of HA increases regardless of the molecular weight used (Figure 4.6). On the other hand, slight inhibition of the contractile behaviour with respect to plasma hydrogels was found when 0.05% HA was incorporated regardless of the molecular weight again (Figure 4.7A). However, no significant differences in contraction with respect to the plasma control were found when this low amount was used. Molecular weight had an impact on contraction of hydrogels by increasing the behaviour as the molecular weight augmented (Figure 4.7B and 4.7C). Overall contraction reached up to 50% when the highest amount of HA was added; 40% for hydrogels with 0.1% of HA content and just 20% whenever 0.05% of HA was present on the hydrogels structure.

Figure 4.4: UV scan from 200 nm to 800 nm of plasma solutions and hydrogels containing A) 0.05% HA (1 MDa); B) 0.1% HA(1 MDa); C) 0.2% HA(1 MDa).

Figure 4.5: Gelation kinetics followed through UV spectroscopy and measured at 315 nm for plasma solutions and hydrogels containing A) 0.05% HA (500 kDa); B) 0.1% HA(700 kDa); C) 0.2% HA(1 MDa)(n=4).

Figure 4.6: Contraction in terms of weight loss for plasma hydrogels containing A) HA 500 kDa MW; B) HA 700 kDa MW ; C) HA 1 MDa MW.

Figure 4.7: Contraction in terms of weight loss for plasma hydrogels containing A) 0.05% of HA; B) 0.1% of HA; C) 0.2% of HA.

4.3.3 Protein release

Release of proteins from the plasma hydrogels with incorporated natural HA was studied through the Bradford assay. As explained in previous chapters, a standard curve was used to extrapolate the absorbance results obtained through UV spectroscopy. The incorporation of natural HA at different concentrations (0.05%, 0.1% and 0.2%) and of different molecular weight (500 kDa, 700 kDa and 1 MDa) was studied yielding as result no significant differences in the profile of the protein release regardless of the amount added or the MW of HA (Figure 4.8).

Figure 4.8: Cumulative protein release for hydrogels containing A) Three concentrations of HA(500 kDa) (0.05%, 0.1% and 0.2%) and B) Three different MWs (500 kDa, 700 kDa and 1 MDa) of HA 0.2%.

4.3.4 Viscosity of natural HA

The viscosity of the three types of natural HA (500 kDa, 700 kDa and 1 MDa) used in these experiments was characterised using a viscosimeter in order to decipher their viscous nature and the effect of this viscosity on the mechanical properties of the plasma hydrogels. It was found that viscosity of the diluted natural HA at 10 mg/ml (1% w/v) increases with the molecular weight. For comparison reasons, plasma viscosity was also measured as control yielding a value similar to that of water, as expected. On the other hand, 500 kDa HA showed a value of viscosity around 50 cP; 700 kDa HA increased that value up to 415 cP and finally 1 MDa HA gave the highest viscosity value, approximately 10,000 cP (Figure 4.9).

Figure 4.9: Viscosity values for the three MWs of HA (500 kDa, 700 kDa and 1 MDa) at 10 mg/ml (1% w/v).

4.3.5 Mechanical characterisation: compression test

The effect of natural HA incorporation on the compressive modulus of elasticity of plasma hydrogels was studied through a uniaxial compression test. Samples of 5 ml final volume and left equilibrated for 24 hours in PBS were measured on an Instron 3366 machine with a 100 N loading cell.

The results obtained when three levels of final HA concentration (0.05%,

0.1% and 0.2%) and three different HA molecular weights (500 kDa, 700 kDa and 1 MDa) were combined in plasma hydrogels was an overall decrease in the compressive modulus of elasticity or Young's modulus (E) for samples containing 0.05% of HA (Figure 4.10A). Samples with HA 500 kDa incorporated always showed a 50% decrease in the mechanical properties (Figure 4.11). Differences between incorporating 0.05% and 0.1% or 0.2% were found in terms of mechanical properties. Higher amounts of HA (0.1% and 0.2%) and high molecular weights (700 kDa and 1 MDa) showed similar mechanical behaviour to the plasma control. Furthermore, increasing the molecular weight increased the compressive modulus of elasticity for all samples progressively regardless of the amount added (Figure 4.10B and 4.10C).

4.3.6 Fibroblast-mediated matrix contraction

Plasma hydrogels containing different amounts (0.05%, 0.1% and 0.2% w/v) and molecular weights (500 kDa, 700 kDa and 1 MDa) of natural HA were prepared containing 80,000 fibroblast/ml of hydrogel. Cells were incorporated in order to assess the area contraction behaviour of hydrogels containing HA in the presence of fibroblast.

The results show that the incorporation of natural HA affects the contraction profile of hydrogels for the first 8 hours of incubation. Increasing the amount of HA incorporated increases the contraction behaviour of hydrogels regardless of the molecular weight used (Figures 4.12, 4.13 and 4.14). This change is more evident in hydrogels containing 500 kDa and 700 kDa natural HA, where, in the latter case the differences in area reached a striking 50% between the samples with 0.2% of HA and the plasma control (Figure 4.13). In this way, hydrogels with higher amounts of natural HA performed worse in terms of contraction for the first 8 hours, but obtained a similar performance after 24 hours incubation and up to the 15 days that the experiment lasted.

4.3.7 Fibroblast proliferation assay

Human primary fibroblast proliferation inside the plasma hydrogels containing natural HA was monitored using the Alamar BlueTM assay, based on the reduction by metabolically active cells of the resazurin reagent into resorufin. The

Figure 4.10: Compressive modulus of elasticity for hydrogels containing the levels of HA MW (500 kDa, 700 kDa and 1 MDa) at HA concentrations A) 0.05%, B) 0.1% and C) 0.2%.

Figure 4.11: Compressive modulus of elasticity for hydrogels containing HA (500 kDa) at three levels of concentration (0.05%, 0.1% and 0.2%).

Figure 4.12: Cell-mediated contraction of plasma hydrogels containing HA (500 kDa) at three concentrations (0.05%, 0.1% and 0.2%).

Figure 4.13: Cell-mediated contraction of plasma hydrogels containing HA (700 kDa) at three concentrations (0.05%, 0.1% and 0.2%).

Figure 4.14: Cell-mediated contraction of plasma hydrogels containing HA (1 MDa) at three concentrations (0.05%, 0.1% and 0.2%).

effect of the natural HA at the defined concentrations (0.05%, 0.1% and 0.2% w/v) and molecular weights (500 kDa, 700 kDa and 1 MDa) was studied at four different time points (1, 3, 7 and 14 days). The same amount of fibroblast (80,000 cells/ml) as in the contraction assay was added to each gel and the fluorescence change monitored at 530 nm excitation wavelength and 590 nm emission wavelength.

The results obtained show a similar proliferation for all conditions with respect to the plasma hydrogels control. Hydrogels containing natural HA of 500 kDa molecular weight show an increase in the proliferative activity with the increasing HA concentration at day 14, with 0.2% samples having a greater proliferative activity than the plasma control (Figure 4.15A). Similar behaviour was found for the hydrogels containing 700 kDa HA, with the proliferation being similar or just below the plasma control samples at 7 and 14 days respectively (Figure 4.15B). For the 1 MDa HA molecular weight case, no significant differences were found between samples containing HA and the control at any time point or condition (Figure 4.15C).

4.4 Discussion

Natural HA purchased from Lifecore® Biomedical and derived from bacterial fermentation was incorporated into the plasma-derived fibrin matrices in order to enhance the mechanical properties and better mimic the ECM formed upon skin damage. HA interacts with fibrinogen in a specific and reversible manner to form reinforced hydrogels with potential capability to serve as regeneration aid in skin damage [15]. Levels of HA increase upon tissue damage, evidencing its role in the wound healing process as explained in the introduction to this thesis [16]. However, the effect of HA in the fibrin matrix needs to be explored in order to render these hybrid matrices useful for skin tissue engineering.

In this chapter, we successfully incorporated the natural HA into the plasma-derived matrices and checked its effect on the gelation time, kinetics, contraction *in vitro* in the presence and absence of cells, viscosity, proteins release, mechanical properties and cell proliferation. Experiments gave mixed results with slight improvements for the hydrogels in some areas (fibroblast proliferation) but lower performance in areas like mechanical properties, gelation time

Figure 4.15: Fibroblasts proliferation embedded in plasma hydrogels containing HA at three MWs (500 kDa, 700 kDa and 1 MDa) and three HA concentrations (0.05%, 0.1% and 0.2%).

and cell-mediated contraction.

Hydrogels formed in the presence of natural HA delayed the polymerization due to the disruption of the process in the presence of HA that is able to interact with the fibrin molecules [17]. Gelation time increased with the increasing MW (500 kDa, 700 kDa, 1 MDa) and concentration (0.05%, 0.1% and 0.2%) of HA except for the HA at 500 kDa, that did not affect gelation independent of the amount of HA added (0.05%, 0.1% and 0.2%) (Figures 4.2 and 4.3). Gelation time can be tuned by means of the crosslinking density as previously reported by Fan *et al.* [18]. In that context, interpenetrating polymeric networks (IPN) arise as a potential solution by tailoring the interaction of two partially intertwined matrices to control better the process [1]. Further characterisation of the gelation process was done through UV-Vis gelation kinetics evidencing the delay in gelation, but the steeper slope obtained suggests a quicker polymerization of the matrices incorporated with natural HA once the fibrinogen specific sites become available (Figure 4.5).

Regarding hydrogels contraction in the absence of human primary fibroblast, the increasing amount of natural HA incorporated increased the mass contraction of hydrogels with time, an undesired outcome due to the loss of material too quickly leading to failure of the hydrogels mechanically (Figures 4.6 and 4.7). The probable reason behind this behaviour is the weak interaction between HA and fibrin making that the material incorporated can potentially escape easily from the disrupted fibrin matrix and thus result in an overall loss of mass with incubation time, although it has not been reported before. Further characterisation of the HA release was not performed for these hydrogels due to the poor results obtained in all areas tested. The release of proteins from the matrices with incorporated natural HA was similar for all the conditions tested evidencing that differences in the mass contraction are independent of the release profile. In this case, proteins inside the matrices are modulating the contraction by delaying it but the presence of HA is main driver for the differences found between the conditions tested.

Mechanical properties studied through uniaxial compression test evidenced the low compressive modulus of elasticity for the hydrogels that contained natural HA (Figure 4.10). These low mechanical performance was expected after the results obtained in the swelling study, with high water loss ratios making the

hydrogels less stable *in vitro*. Even though mechanical properties were shown to be worse than the plasma counterparts, cell-mediated contraction was not greatly affected by the presence of natural HA, with hydrogels performing in a similar way as the controls (Figures 4.12, 4.13 and 4.14). Natural HA of 700 kDa MW was the condition that made the hydrogels contract more in the first hours, due to the higher affinity for fibroblast to attach to the matrix in the presence of a more similar ECM composition.

The final study performed was the human primary fibroblast proliferation assay inside the matrices. Results show an increase in metabolic activity of cells as the amount of HA increased from 0.05% to 0.2%, with this last final concentration of natural HA obtaining better proliferation than the plasma control (Figure 4.15). Although the performance of cell proliferation inside the HA-Fibrin matrices was shown to be equal or even slightly higher than plasma alone, the poor results obtained for the rest of the experiments and the main driver of this thesis of improving the matrices mechanically lead us to try to find an alternative reinforcement material to enhance the performance of human plasma-derived matrices for skin tissue engineering.

In that context, the possibility of using a chemically modified HA that could be crosslinked and thus could better stabilise the plasma matrix was explored. In the next chapter, thiol-modified HA crosslinked with PEGDA was incorporated into the plasma-derived fibrin hydrogels and their performance assessed in order to overcome the main issues related with plasma hydrogels stated in this chapter.

Bibliography

- [1] F. Lee and M. Kurisawa, "Formation and stability of interpenetrating polymer network hydrogels consisting of fibrin and hyaluronic acid for tissue engineering," *Acta Biomaterialia*, vol. 9, pp. 5143–5152, feb 2013.
- [2] H.-J. Stark, K. Boehnke, N. Mirancea, M. J. Willhauck, A. Pavesio, N. E. Fusenig, and P. Boukamp, "Epidermal homeostasis in long-term scaffold-enforced skin equivalents," in *Journal of Investigative Dermatology Symposium Proceedings*, vol. 11, pp. 93–105, Elsevier, 2006.
- [3] W. Y. J. Chen and G. Abatangelo, "Functions of hyaluronan in wound repair," *Wound repair and regeneration*, vol. 7, no. 2, pp. 79–89, 1999.
- [4] S. A. Meyers, A. V. Seaber, R. R. Glisson, and J. A. Nunley, "Effect of hyaluronic acid/chondroitin sulfate on healing of full-thickness tendon lacerations in rabbits," *Journal of orthopaedic research*, vol. 7, no. 5, pp. 683–689, 1989.
- [5] D. A. R. Burd, M. Ritz, S. Regauer, M. T. Longaker, J. W. Siebert, and H. Garg, "Hyaluronan and wound healing: a new perspective," *British journal of plastic surgery*, vol. 44, no. 8, pp. 579–584, 1991.
- [6] K. R. Kirker, Y. Luo, J. H. Nielson, J. Shelby, and G. D. Prestwich, "Glycosaminoglycan hydrogel films as bio-interactive dressings for wound healing," *Biomaterials*, vol. 23, no. 17, pp. 3661–3671, 2002.
- [7] J. M. Estes, N. S. Adzick, M. R. Harrison, M. T. Longaker, and R. Stern, "Hyaluronate metabolism undergoes an ontogenic transition during fetal development: implications for scar-free wound healing," *Journal of pediatric surgery*, vol. 28, no. 10, pp. 1227–1231, 1993.
- [8] K. Rolfe and A. Grobbelaar, "A review of fetal scarless healing," *ISRN dermatology*, vol. 2012, 2012.
- [9] "Research Grade Sodium Hyaluronate - Lifecore. Retrieved from <https://www.lifecore.com/sodium-hyaluronate/research-grade-sodium-hyaluronate/>. Access date: 2019-06-11."
- [10] R. J. Faust, "Technical Manual of the American Association of Blood Banks," *Anesthesiology*, vol. 65, no. 2, p. 242, 1986.

- [11] J. L. Vanderhooft, M. Alcoutlabi, J. J. Magda, and G. D. Prestwich, "Rheological Properties of Cross-Linked Hyaluronan–Gelatin Hydrogels for Tissue Engineering," *Macromolecular bioscience*, vol. 9, no. 1, pp. 20–28, 2009.
- [12] B. Jeong, Y. H. Bae, and S. W. Kim, "Thermoreversible gelation of peg-plga- peg triblock copolymer aqueous solutions," *Macromolecules*, vol. 32, no. 21, pp. 7064–7069, 1999.
- [13] "Viscometer NDJ 8S | DHgate.Com. Retrieved from: <https://es.dhgate.com/product/digital-rotary-viscometer-ndj-8s-viscosimeter/434596430.html>. Access date: 2019-09-09."
- [14] Student, "The probable error of a mean," *Biometrika*, pp. 1–25, 1908.
- [15] R. D. LeBoeuf, R. R. Gregg, P. H. Weigel, and G. M. Fuller, "Effects of hyaluronic acid and other glycosaminoglycans on fibrin polymer formation," *Biochemistry*, vol. 26, no. 19, pp. 6052–6057, 1987.
- [16] V. N. Khabarov, P. Y. Boykov, and M. A. Selyanin, *Hyaluronic Acid: Production, Properties, Application in Biology and Medicine*. John Wiley & Sons, 2014.
- [17] J. R. E. Fraser, T. C. Laurent, and U. Laurent, "Hyaluronan: its nature, distribution, functions and turnover," *Journal of internal medicine*, vol. 242, no. 1, pp. 27–33, 1997.
- [18] M. Fan, Y. Ma, J. Mao, Z. Zhang, and H. Tan, "Cytocompatible in situ forming chitosan/hyaluronan hydrogels via a metal-free click chemistry for soft tissue engineering," *Acta biomaterialia*, vol. 20, pp. 60–68, 2015.

Incorporation of thiol-modified HA crosslinked with PEGDA in plasma-derived fibrin hydrogels

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5.1 Introduction

There is an increasing need to generate more robust and mechanically sound matrices for the regeneration of skin after severe damage. This need has lead this research group to seek for potential solutions to early shrinkage, increased contraction and poor stability by the incorporation of new materials into the already well characterised plasma-derived fibrin matrix. In chapter 4 we have presented hyaluronic acid (HA) as an ideal candidate to overcome all the mentioned drawbacks of the fibrin matrices nowadays. After characterising the incorporation of an unmodified, natural HA into the plasma hydrogels and obtaining mixed results, we decided to shift our focus into chemically modified HA that could form a parallel matrix via crosslinking with an also chemically modified PEG in the form of polyethylene (glycol) diacrylate (PEGDA). This approach seemed to be the right decision since crosslinking the HA would better maintain the material inside the matrices and thus enhance their mechanical and biological properties, key aspects for the correct performance of the hydrogels.

In this chapter, the incorporation of HA-PEGDA into the plasma-derived fibrin hydrogels will be studied by means of gelation time and kinetics, contraction both with and without cells, protein release, HA release from the

matrices, mechanical and structural characterisation, cellular (fibroblast and keratinocytes) proliferation in the presence of HA-PEGDA and finally generation of organotypic skin constructs *in vitro*.

As explained in previous chapters, high contraction rates and low mechanical properties are limiting the application of plasma-derived matrices as both testing devices and transplantation alternatives [1]. The addition of thiol-modified HA crosslinked with PEGDA opens the possibility of enhancing the mechanical properties and decreasing the contraction rate in order to generate matrices with better handling for the surgeons and capable of generating good clinical results after transplantation.

5.2 Materials & Methods

5.2.1 Materials

Materials used in this chapter include the ones previously described in section 2.2.1 of Materials & Methods Chapter 2. Frozen human plasmas with low content of platelets were obtained from a blood bank in Spain (Banco de Sangre del Centro Comunitario de Transfusión del Principado de Asturias (CCST)) and treated according to the standards of the American Association of Blood Banks [2]. HyStem commercially available hydrogels system consisting on Glycosil Hyaluronic Acid (HA) and Extralink[®] polyethylene (glycol) diacrylate (PEGDA) were obtained from ESI Bio (Biotime, Inc.) [3]. Antifibrinolytic agent Amchafibrin (Tranexamic acid 500 mg, Meda Pharma SL, Spain), sodium chloride (NaCl 0.9%) and calcium chloride (CaCl₂ 1%)(Sigma Aldrich, USA) were used for hydrogels formation. Phosphate-buffered saline (PBS) (Sigma Aldrich, USA) was used to incubate hydrogels. Bradford assay reagent (Bio-Rad Protein Assay Dye Reagent Concentrate) (Bio-Rad, Germany) and Bovine serum albumin (BSA) (1 mg/ml) (Sigma Aldrich, USA) were used to measure protein release. Hyaluronic Acid Sandwich ELISA kit purchased from Echelon Biosciences, USA was used to quantify the HA release. Different dilutions of pure ethanol were used to dehydrate hydrogels for structural characterisation. As cell culture medium, Dulbecco's Modified Eagle's Medium (DMEM) + GlutaMAX[™] supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin (P/S) (Thermo Fisher Scientific, USA) were

used. Keratinocyte culture medium composed of: Ham's F-12 nutrient mix, GlutaMAXTM, from Life Technologies, USA; Primocyn antibiotic, FCS HII-Clon, Hydrocortisone, Adenine, Insulin, T3, Ascorbic acid, choleric toxin and epidermal growth factor (EFG) were obtained from Thermo Fisher Scientific, USA. Cellular proliferation and viability was measured with Alamar BlueTM Cell Viability Reagent and Ibidi[®] μ -slide 8 well glass bottom were used for confocal microscopy visualization of hydrogels in the Live/Dead[®] Cell Assay (Thermo Fisher Scientific, USA). Keratinocyte proliferation was measured in special medium CNT57 obtained from Cellntec, Spain. MTS Reagent for cell viability assay was purchased to Sigma Aldrich, USA. Falcon[®] 6 Well Deep from Cultek, Spain and Transwell permeable Falcon[®] 6-well plates were used to generate organotypic *in vitro* skins. Immunohistochemical characterisation of the skins was made with the following antibodies purchased to Thermo Fisher Scientific: COL7A1 Monoclonal Antibody (LH7.2), Cytokeratin 5 Monoclonal Antibody (XM26), Cytokeratin 10 Monoclonal Antibody (DE-K10), Vimentin Monoclonal Antibody (V9), Filaggrin Monoclonal Antibody (FLG01).

5.2.2 Plasma-derived hydrogels containing thiolated HA crosslinked with PEGDA preparation

The protocol followed to generate plasma hydrogels with thiolated hyaluronic acid crosslinked with PEGDA was the same as described in section 2.2.2 of Materials & Methods – Chapter 2. The only change was the incorporation of the new materials in a dissolved state. Modified hyaluronic acid containing thiol functional groups was purchased to ESI Bio (Biotime, Inc.) [3] in the form of a hydrogel kit containing also the necessary PEGDA to crosslink the material. The kit consisted on 6 vials containing 10 mg of thiolated HA and 3 vials containing 2.5 mg of PEGDA. HA vials were dissolved in 1 ml of sterile 0.9% NaCl while PEGDA vials were dissolved in 200 μ l of CaCl₂. HA needed of agitation and heat (37°C) for 30 minutes in order to fully dissolve and be ready to use.

In order to make the hydrogels containing thiolated HA, the necessary amount of NaCl was removed in order to accommodate the new material. Same procedure was followed with PEGDA, in this case subtracting the pre-calculated amount needed to the total amount of CaCl₂. Human plasma hydrogels were then prepared maintaining a final fibrinogen concentration of 1.2 mg/ml, 0.008%

w/v of Amchafibrin and 0.08% CaCl_2 . HA final concentration inside the hydrogels varied between three different physiological levels: 0.05%, 0.1% and 0.2% of w/v ratio [4]. In order to incorporate PEGDA at an amount related to the quantity of HA, the mole ratio between thiol groups present in HA and the double bond groups in PEGDA was used as reference. In this way, four different ratios of crosslinking were defined: 2:1, 6:1, 10:1 and 14:1 (thiol: double bond). Finally the appropriate amounts of NaCl (0.9%) and CaCl_2 (1%) were added to get to the final volume of each hydrogel. All hydrogels were left incubating for 1 hour at 37°C and 5% CO_2 for their complete gelation.

5.2.3 Gelation time and kinetics

The effect of the incorporation of thiolated HA crosslinked with PEGDA in the gelation time and kinetics was studied as reported in section 2.2.3 Materials & Methods Chapter 2. The classical flip-flop method and a turbidimetry assay were performed in plasma hydrogels containing different amounts of HA (0.05%, 0.1% and 0.2% w/v) and four levels of PEGDA crosslinking (2:1, 6:1, 10:1 and 14:1) as explained in the previous section. In short, hydrogels were prepared in 19 mm diameter glass-vials and incubated at 37°C and 5% CO_2 for 1 hour. Vials were visually inspected every 30 second in order to determine the time at which no liquid is left inside the vials.

Furthermore, turbidimetry assay was performed in hydrogels containing thiolated HA previously prepared in a glass 96-well plate. First, a UV scan covering the whole spectrum from 200 nm to 800 nm was performed in order to determine the wavelength at which the maximum difference in O.D. was found. Later, UV gelation experiment was performed at the designated wavelength with samples being measured every 30 seconds inside the SynergyTM HTX Multi-Mode Microplate Reader at 37°C in order to obtain the gelation kinetics profile of the hydrogels.

5.2.4 Hydrogels contraction in the presence of thiolated HA crosslinked with PEGDA

Changes in weight for plasma-HA hydrogels during incubation in PBS were measured following the previously described procedure in section 2.2.4 Materi-

als & Methods Chapter 2. Briefly, hydrogels were detached from the glass-vials where they were prepared and quickly weighted in a precision scale. Hydrogels containing three concentrations of thiolated HA (0.05%, 0.1% and 0.2% w/v) and four levels of PEGDA crosslinking (2:1, 6:1, 10:1 and 14:1) were incubated at 37°C for 72 hours in PBS and their weight measured at a precision scale at each time point (0h, 1h, 2h, 4h, 6h, 8h, 24h, 48h and 72h). Swelling ratio of the hydrogels was then calculated with the following equation 5.1.

$$SR_W = \frac{W_T}{W_0} \quad (5.1)$$

5.2.5 Protein release

Protein release from the hydrogels prepared with thiolated HA and PEGDA crosslinker was studied through the Bradford assay as explained in section 2.2.5 of Material & Methods Chapter 2. PBS samples at different time points (0h, 1h, 2h, 4h, 6h, 8h, 24h and 48h) were obtained from the surrounding medium while incubation took place and were later diluted and mixed with Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad, USA). This mixture was measured for the change in absorbance at 595 nm in the Biowave II spectrophotometer (Biochrom, UK). The total amount of proteins released from the hydrogels was calculated extrapolating the data from a previously calculated standard curve (Figure 2.14) and taking into account the dilution factors applied.

5.2.6 HA release from hydrogels

The potential release of the incorporated hyaluronic acid from the plasma hydrogels was assessed through an HA sandwich ELISA assay from Echelon Biosciences, USA, specific for the detection of hyaluronic acid in blood serum samples. Hydrogels were prepared in 19 mm diameter glass vials and transferred to p-35 culture dishes after 1 hour of gelation inside the incubator at 37°C and 5% CO₂. Hydrogels containing two different crosslinking ratios (14:1 and 2:1) of thiolated hyaluronic acid and PEGDA were tested with three different final concentrations of HA (0.05%, 0.1% and 0.2% w/v). Hydrogels were incubated with PBS for 24 hours before 100 µl samples of the surrounding medium where

taken to make the assay.

Samples of the surrounding medium had to be diluted 1:100 in order to fall inside the generated HA calibration curve (Figure 5.1). The protocol followed was the one that came inside the commercial kit and consisted on the detection of HA of minimum size 100 kDa. Results were extrapolated using the standard curve as reference using equation 5.2 and the total amount of HA released from the hydrogels calculated.

Figure 5.1: HA standard curve generated experimentally from the HA sandwich ELISA.

$$y = 0.327 \log X - 0.3583 \quad (5.2)$$

5.2.7 Mechanical characterisation: compression test

The mechanical properties of plasma-derived hydrogels with incorporated thiolated hyaluronic acid were studied through a compression test carried out with an Instron 3366 machine. As explained in section 2.2.6 of Materials & Methods Chapter 2, hydrogels were prepared in 25 mm diameter glass vials 24 hours

prior to the mechanical test and maintained in PBS at 37°C before taking them to ICTP. The conditions studied were three different HA final concentrations inside the gel (0.05%, 0.1% and 0.2% w/v) and four different crosslinking ratios for each HA concentration (2:1, 6:1, 10:1 and 14:1 thiol to double-bond moles ratio). Results were expressed in terms of the stress/strain curve and the compressive modulus of elasticity or Young's modulus (E) at 20% strain.

5.2.8 Structural characterisation (SEM)

The structural characterisation of plasma hydrogels containing thiolated HA and PEGDA was obtained through the visualization of their cross-section in the scanning electron microscope as explained in section 2.2.7 of Material & Methods Chapter 2. Briefly, plasma hydrogels were prepared in 19 mm diameter glass-vials with a final volume of 3 ml. After 1-hour hydrogel formation, they were detached and transferred to 20% ethanol solution in order to progressively dehydrate the sample for their critical CO₂ point drying. A series of ethanol dilutions (20%, 40%, 60%, 80% and 100%) were used in order to exchange the water with ethanol in a process that lasted 48 hours. Samples were then dried at the supercritical CO₂ reactor at CSIC at the following parameters resumed in table 5.1.

Time (min)	Temperature (°C)	Pressure (bar)
10	35	0-100
90	35	100
30	35	100-0

Table 5.1: Supercritical CO₂ drying conditions.

Plasma hydrogels with incorporated thiolated HA were then sectioned with the aid of liquid nitrogen, mounted and gold sputtered for their visualization under the SEM. Images were obtained at different magnifications (5,000X, 10,000X and 20,000X) and the fibres diameters calculated using ImageJ software.

5.2.9 Cell culture

As explained in section 2.2.8 of Materials & Methods Chapter 2, human primary fibroblast obtained from skin biopsies were used to perform the cellular experiments involving the encapsulation of cells inside the plasma-derived fibrin matrices. Fibroblasts were stored in liquid nitrogen at -195.8°C and defrost and seeded into T75 flask before the start of each experiment. Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin/Streptomycin (P/S) antibiotic/antimycotic agent was used as culture medium. Cells were maintained at 37°C and 5% CO_2 pressure in the incubator and medium was changed every 2 days until fibroblast reached confluence.

Detachment from the T75 flask proceeded as follows; pre-warmed PBS was added into the culture flask after medium was vacuumed in order to remove any excess of supplemented DMEM. Two millilitres of trypsin were added into the flask and left incubation for 10 minutes at 37°C in order to detach the cells from the bottom. The flask was visualised under the microscope to assure all the fibroblast were floating and trypsin was inactivated by adding supplemented medium on top. Fibroblast were then counted by putting 10 μl of the solution into a Neubauer chamber and counting under the microscope in order to assess the number of cells. The solution containing the fibroblast was then centrifuged for 7 minutes and 1200 rpm after which the supernatant was discarded and the pellet of cells was re-suspended in the desired amount of supplemented medium to perform the experiment.

In order to generate the dermo-epidermal skin constructs, human primary keratinocytes were used on top of the constructs to form the epidermal layer. This type of cells are incapable of growing *in vitro* in the absence of a feeder layer of cells that in this case was conformed by irradiated mouse embryonic fibroblast (3T3 $\pm$ J2/Swiss albino mouse 3T3). This irradiated layer of cells is incapable to proliferate thus allowing the primary keratinocytes to form colonies and grow using the factors generated by the feeder cells. The culture medium used for keratinocytes contained a 3:1 mixture of DMEM and HAM's F12, supplemented with 10% FBS, 8 ng/ml choleric toxin, 2 nM T3, 0.4 mg/ml hydrocortisone, 5 mg/ml insulin, 10 ng/ml EGF and 25 $\mu\text{g}/\text{ml}$ adenine. Human primary keratinocytes were detached from the T75 flask following the previously explained protocol with an incubation in trypsin at 37°C for 20 minutes.

5.2.10 Fibroblast-mediated matrix contraction

The effect of thiolated HA crosslinked with PEGDA on the contractile behaviour of human plasma hydrogels in the presence of primary fibroblast was studied through an area contraction study as explained in in section 2.2.9 of Materials & Methods Chapter 2. Human primary fibroblast were incorporated into the hydrogels at 80,000 cells/ml density and three levels of HA concentration (0.05%, 0.1% and 0.2%) and four levels of PEGDA crosslinking (2:1, 6:1, 10:1 and 14:1) were studied. Hydrogels were prepared in 19 mm diameter glass-vials and initial area was defined as the area after detachment of the hydrogels. Hydrogels were incubated in supplemented medium (DMEM, 10% FBS, 1% P/S) and images of the surface area obtained with pictures taken at different time points (0h, 4h, 6h, 8h, 24h, 48h, 72h, 7 days, 10 days and 15 days). The surface area contraction was calculated with ImageJ using the following equation 5.3.

$$SR_A = \frac{A_T}{A_0} \quad (5.3)$$

Furthermore, a cell-mediated contraction index was defined to better show the behaviour of the modified HA hydrogels with respect to the plasma controls. The index was calculated with the following equation 5.4.

$$\text{Contraction Index} = \frac{(A_0 - A_i)_E - (A_0 - A_i)_C}{(A_0 - A_i)_C} \quad (5.4)$$

Where $(A_0 - A_i)_E$ is the difference between initial area ($t=0$) and final area at a given time-point of experimental and $(A_0 - A_i)_C$ is the difference between initial area ($t=0$) and final area at any given time-point of control.

Negative contraction indexes thus represent that gels contracted less than the control, while positive indexes mean higher contraction of experimental than control.

5.2.11 Fibroblast proliferation assay

The effect of the incorporation of thiolated HA crosslinked with PEGDA on the proliferation of human primary fibroblast inside the plasma matrices was studied through the Alamar BlueTM assay as previously explained in section 2.2.10 of Materials & Methods Chapter 2. The hydrogels with three levels of HA concentration (0.05%, 0.1% and 0.2% w/v) and four levels of PEGDA crosslinking (2:1, 6:1, 10:1 and 14:1) were prepared containing 80,000 cells/ml in black 96-well plates with a final volume of 100 μ l per gel. The plates were left incubating at 37°C and 5% CO₂ for 1 hour to allow for the complete gelation of the matrices. Supplemented medium containing DMEM with 10%FBS and 1% P/S was later added to each well and the plates were left incubating before being measured for proliferation at time points 1 day, 3 days, 7 days and 14 days.

In order to measure the fibroblast metabolic activity inside the hydrogels, medium containing 10% Alamar BlueTM cell viability reagent was added to each well. The plate was incubated for 3 hours in order for the cells to reduce the active reagent indicating the proliferation of cells inside the matrices. The change in fluorescence intensity of the Alamar BlueTM cell viability reagent was recorded with the SynergyTM HTX Multi-Mode Microplate Reader at 570 nm excitation and 600 nm emission wavelengths. Data was processed to express the level of fluorescence found for each HA condition.

5.2.12 Live/Dead assay

Human primary fibroblast viability inside the thiolated hydrogels was further characterised with the classical Live/Dead[®] Viability/Cytotoxicity kit for mammalian cells assay as explained in section 2.2.11 of Materials & Methods Chapter 2. Hydrogels containing thiolated HA were prepared in μ -slide 8-well glass bottom plates with a final volume of 100 μ l per gel. A total of 80,000 cells/ml were studied for each condition (HA at 0.05% w/v and 2:1 and 6:1 crosslinking ratios). Gelation was allowed to occur for 1 hour at 37°C and 5% CO₂ before adding 200 μ l of cell culture medium containing 10% FBS and 1% P/S. Plasma control hydrogels were also prepared without HA and gels were left incubating for 48 hours before performing the Live/Dead assay.

Live dead staining was performed by adding calcein AM at a concentration

of 0.5 $\mu\text{l/ml}$ of PBS and ethidium homodimer with final concentration of 2 $\mu\text{l/ml}$ of PBS and incubating the mixture with the hydrogels for 30 minutes at 37°C. After the incubation period, excess of the reagents was washed with PBS and the plate covered with aluminium foil was transported to the confocal microscope for their visualization. Stack of images in the z-axis was obtained and reconstructed using ImageJ to fully characterise the viability of human primary fibroblast inside the hydrogels containing thiolated HA.

5.2.13 Keratinocyte proliferation assay

Proliferation capacity of human primary keratinocytes was assessed as previously described in section 2.2.12 of Materials & Methods Chapter 2 with the MTS assay. Hydrogels containing the three levels of crosslinking (2:1, 6:1, 10:1 and 14:1) and three different HA concentrations (0.05%, 0.1% and 0.2% w/v) were prepared in 96-well plates in the absence of human primary fibroblast so that no interference in the quantification occurred. Special conditioned medium CNT57 was used and final volume of 100 μl per gel was prepared. Hydrogels were left incubating for 2 hours at 37°C and 5% CO₂ in order for the gelation process to fully occur in the absence of fibroblast. Conditioned medium CNT57 was then added on top of the gels in order to wash out the excess of calcium and left for 24 hours. Primary keratinocytes previously cultured until confluence were detached and seeded on top of the hydrogels at a density of 100,000 cells/ml of hydrogel. Constructs were left for 48 hours untouched in order for the keratinocytes to attach. Proliferation was then measured at three different time points (1, 4 and 7 days) by incubating the hydrogels with medium containing a 10% MTS reagent for 2 hours and measuring the change in absorbance at 490 nm at the SynergyTM HTX Multi-Mode Microplate Reader.

5.2.14 Organotypic skin generation with thiol-modified HA crosslinked with PEGDA

The final step of this thesis was to validate the ability of plasma-derived fibrin hydrogels containing thiolated HA to generate fully formed *in vitro* skin constructs. Using the already established protocol first developed by Meana et al. [5] and Llames et al. [6], the aim was to enhance the performance of the organotypic skin constructs.

Two types of cells are needed in order to generate these skin equivalents: human primary fibroblast and human primary keratinocytes. Culture conditions for the fibroblast consist on their expansion by using DMEM supplemented with 10% FBS and 1% P/S. However, primary keratinocytes need of a feeder layer in order to proliferate *in vitro*. This feeder layer consist of mouse irradiated fibroblast that are unable to proliferate due to the damage in their DNA but that are capable of secreting the necessary factor for human primary keratinocytes culture. Apart from the feeder layer, special cell culture medium with specific factor is also needed. This medium is composed of DMEM, F12, Insulin, Primocyn, Adenin, Hidrocortisone, Epiderma Growth Factor (EFG), Tossina Colerica, Triiodotironina and 10% HII clone serum.

Both types of cells were cultured until they achieved confluence before proceeding to make the skin constructs. Plasma hydrogels of known fibrinogen concentration were prepared as explained in section 5.2.2 with a final volume of 4 ml. Fibroblast were incorporated into the hydrogels matrix at a concentration of 20,000 cells/ml and HA at a final concentration of 0.05% and 0.1% w/v ratio. Two different crosslinking ratios were studied, 2:1 and 6:1 thiol to double-bond moles. Hydrogels were generated in transwell culture inserts and incubated in 6-well plates for 1 hour, 37°C and 5% CO₂. After gelation took place, excess of calcium had to be removed by incubating the hydrogels with supplemented medium at the bottom of the plate and at the top of the gel for 24 hours.

Once calcium was removed, medium was removed with the vacuum system and the keratinocytes were seeded on top of the plasma-HA hydrogels at a final amount of 1.5 million cells/well. Human primary keratinocytes needed 48 hours to attach to the surface of the hydrogels. After this period of time, hydrogels were visually inspected under the microscope in order to check the confluence of the keratinocytes seeded on top. Once the keratinocytes had formed a uniform confluent layer of cells covering all the surface of the hydrogel, these were ready to be cultured in an air-liquid interface. In order to achieve that, medium covering the keratinocytes was removed and the transwells transferred to deepwell plates. Surface of the gels needed to be completely dry in order to achieve a correct differentiation of the keratinocytes and thus a correct skin formation. Five minutes were allowed under the laminar flow of the cell culture cabin for the hydrogels to completely dry. After this period of time, differentiation medium

containing all the factors of keratinocyte culture medium but only 0.5% of HII clon serum was added to the bottom of the deepwell plates and the construct were incubated at 37°C and 5% CO₂.

Organotypic skin cultures were visually inspected every day and differentiation medium was changed whenever the colour was turning yellow. Good indicatives of the correct differentiation are the rapid consumption of the medium and the contraction in the Z-axis of the skin constructs. After 18 days of incubation, skin equivalents were taken off the incubator and processed for histological characterisation.

5.2.15 Histological characterisation

Histological characterisation proceeded as follows: transwells were cut through the bottom with the aid of a scalper and the skin constructs placed over tissue paper. Each of the constructs was cut in half in order to fix one half with formaline and the other half with tissue freezing medium. Formaline samples were left for 24 hours inside cassettes and then submerged in 4°C PBS where they were left before being processed at Ciemat facility. Frozen samples were first stored at -20°C and then transferred to -80° refrigerator.

Once all the samples had been processed and mounted into paraffin blocks, microtome machine was used to generate 5 µm slices of the fixated tissues. These slices were transferred to glass holders, which were first processed for hematoxylin-eosin (H&E) staining. After the H&E staining, samples were visually inspected to check for the correct structure of the skin before making a further characterisation with specific antibodies against normal skin proteins.

Immunohistochemistry technique was used to make a profound characterisation of the organotypic skin generated *in vitro*. Typical markers for correct skin formation are vimentin (Vimentin Monoclonal Antibody V9, Thermo Fischer Scientific, IL, USA) for the staining of human fibroblast; cytokeratin 5 (Cytokeratin 5 Monoclonal Antibody XM26, Thermo Fischer Scientific, IL, USA) for the characterisation of basal human keratinocytes; cytokeratin 10 (Cytokeratin 10 Monoclonal Antibody DE-K10, Thermo Fischer Scientific, IL, USA) labelling of suprabasal keratinocytes; collagen VII (COL7A1 Polyclonal Antibody LH7.2, Thermo Fischer Scientific, IL, USA) for the detection of the dermo-epidermal

junction and filaggrin (Filaggrin Monoclonal Antibody, FLG01, Thermo Fischer Scientific, IL, USA) that stains the epidermal granular layer [7, 8]. Paraffin embedded samples were stained with all antibodies in order to fully characterise the correct skin formation *in vitro* in the presence of thiolated hyaluronic acid.

Sandwich technique was used for the immunohistochemistry analysis of the samples, using as secondary antibody Biotin-SP-conjugated AffiniPure Donkey Anti-Mouse IgG (H+L)(Jackson ImmunoResearch Europe Ltd, UK).

Immunohistochemistry proceeded as follows. Samples were deparaffinized with a series of histoclear (xylene) and ethanol dilutions. After removing the paraffin, samples were incubated with methanol containing 1% v/v of H_2O_2 for ten minutes and blocked with 3% BSA-PBS for 1 hour. Primary antibodies were then diluted at the proper concentration (Table 5.2) in 3% BSA-PBS and 30 μ l were added per sample, which were left overnight incubating in pre-humidified dark chambers at 4°C. The secondary biotinilated antibody (Biotin-SP-conjugated AffiniPure Donkey Anti-Mouse IgG (H+L)) diluted 1:250 in 3% BSA-PBS was then incubated with the samples for 45 minutes. Next step was a 1 hour incubation of the samples with the ABC solution inside the dark humidified chambers at ambient temperature. Finally, samples were revealed with DAB kit under the microscope, checking the oxidation of the samples. Hematoxylin staining was then applied to the samples and they were mounted and coverslipped with the aid of Mowiol.

Antibody	Dilution
Vimentin	1:100
Keratin 5	1:20
Keratin 10	1:400
Filaggrin	1:100
Collagen VII	1:2500

Table 5.2: Antibodies and dilutions used for immunohistochemical staining.

5.2.16 Statistical analysis

Statistical analysis was performed using IBM[®] SPSS software. All the data averages and standard deviations were subjected to Student's t-test [9] in order

to determine potential statistical difference significance in the results. Three levels of significance were assessed: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

5.3 Results

5.3.1 Gelation time and kinetics

The effect of the incorporation of thiolated hyaluronic acid crosslinked with PEGDA in the gelation time and kinetics of the plasma-derived hydrogels was assessed through the flip-flop method and a turbidimetry assay. The flip-flop method yielded as result an increase in the gelation time with the increasing amount of HA (0.05%, 0.1% and 0.2% w/v) regardless of the level of crosslinking ratio (2:1, 6:1, 10:1 and 14:1) (Figure 5.2). The biggest change was found in the hydrogels with 0.2% of HA that reached gelation times of 25.3 ± 4.0 minutes (HA 0.2% - 10:1) compared to the plasma hydrogels (13.4 ± 2.4 minutes).

Regarding the turbidimetry assay, results show that the change in O.D. from the liquid to the hydrogel state is mainly due to the plasma component (Figure 5.3A), with a minimal change recorded for the HA-PEGDA hydrogels alone (Figure 5.3B). This is evidenced in the UV scans performed to assess the correct UV wavelength at which the difference in O.D. for liquid vs. gel is maximum. From the data, 325 nm wavelength was selected to perform the UV gelation kinetics experiment

However, an effect of the incorporation of thiolated HA crosslinked with PEGDA was found in the UV gelation kinetics of the hydrogels. Results shown in figure 5.4 evidence that the maximum O.D. reached by the hydrogels when different amounts of HA and crosslinking levels with PEGDA are added is lower than the O.D. reached by plasma hydrogels alone (Figure 5.4). More specifically, adding 0.2% of HA for 6:1, 10:1 and 14:1 ratios was found to be the condition that gave the lowest levels of O.D. Furthermore, crosslinking at 2:1 ratio gave as result very little difference in O.D. at 325 nm between the liquid and gel state for HA 0.1% and 0.2% (Figure 5.4A). This effect is explained as the structure of the HA-PEGDA hydrogel confers a higher degree of transparency to the plasma hydrogels as evidenced in the lower O.D. reached for these types of gels. These HA-PEGDA hydrogels can have different levels of crosslinking

Figure 5.2: Gelation times with the flip-flop method for plasma hydrogels containing three concentrations of thiolated HA (0.05%, 0.1% and 0.2%) and crosslinked at A) 2:1 ratio; B) 6:1 ratio; C) 10:1 ratio; D) 14:1 ratio.

(2:1, 6:1, 10:1 and 14:1) being the 2:1 the highest level of crosslinking and thus explaining the reason behind the lower levels of O.D. found in the HA 0.1% 2:1 and HA 0.2% 2:1 hydrogels (Figure 5.4A).

5.3.2 Hydrogels contraction in the presence of thiolated HA crosslinked with PEGDA

The contractile behaviour of plasma-derived hydrogels containing thiolated HA and PEGDA was studied through a mass swelling experiment. Hydrogels were

Figure 5.3: UV-Vis scan from 200 nm to 800 nm of A) Plasma hydrogels at 1.2 mg/ml; B) HA-PEGDA hydrogel following the commercial formula.

Figure 5.4: Gelation kinetics measured through UV spectroscopy at 325 nm for plasma hydrogels containing three concentrations of thiolated HA (0.05%, 0.1% and 0.2%) and crosslinked at A) 2:1 ratio; B) 6:1 ratio; C) 10:1 ratio; D) 14:1 ratio.

incubated for 72 hours in PBS at 37°C and their change in weight monitored at different time points (0h, 1h, 2h, 4h, 6h, 8h, 24h, 48h and 72h). This experiment was performed to gain an insight on the effect of HA on the matrices

and seeking the reduction of the natural contraction found previously as a consequence of the fibrin matrix as explained in Chapter 3.

The results show that for all crosslinking levels of PEGDA (2:1, 6:1, 10:1 and 14:1) a lower loss of weight was found with the increasing amount of HA (0.05%, 0.1% and 0.2%)(Figures 5.5, 5.6, 5.7 and 5.8). For all conditions, mass loss was mainly produced in the first 8 hours of incubation. In the case of 2:1 crosslinking ratio, adding 0.05% and 0.1% of HA reduced the contractile behaviour about 5%; while when 0.2% of thiolated HA was added a whopping 25% decrease in mass loss was found with respect to the plasma control (Figure 5.5). The same behaviour was found at 6:1 ratio, with 5% decrease for 0.05% HA and 0.1% HA while an still impressive 20% reduction in contraction was found for the hydrogels containing 0.2% of HA in their structure (Figure 5.6). This effect was less evident when the crosslinking ratios used were decreased to 10:1 and 14:1 PEGDA. A reduction in mass loss was found for 10:1 at all HA quantities for the first 8 hours, with HA 0.05% 10:1 being the condition that less mass lost at 12% (Figure 5.7). On the other hand, 14:1 crosslinking ratio yielded as result a clear decrease in mass loss of around 10% at all time-points for the hydrogels containing 0.05% HA and 0.1% HA; while the condition HA 0.2% 14:1 showed a similar behaviour to the plasma control hydrogel, maybe due to such low level of crosslinking (Figure 5.8).

5.3.3 Protein release

The effect of the incorporation of thiolated HA crosslinked with PEGDA on the release of proteins from the plasma-derived hydrogels was studied as previously reported through the Bradford assay. Release of proteins was complete after 24 hours of incubation for all samples.

The results obtained show an increase in the proteins released for HA 0.05% at all crosslinking levels (6:1, 10:1, 14:1 and no crosslinker)(Figure 5.9A); while a decrease in the amount of proteins released to the medium was found when the hydrogels contained high amounts of HA (0.1% and 0.2%) regardless of their crosslinking level (6:1, 10:1, 14:1 and no crosslinker)(Figure 5.9B and 5.9C). Apparently, no effect in the retention of proteins is obtained when low amounts of HA are present in the plasma hydrogel structure, this might be due to the amount of HA not being enough to retain the proteins and favouring the release

Figure 5.5: Contraction of plasma hydrogels in terms of weight loss containing three concentrations of thiolated HA (0.05%, 0.1% and 0.2%) and crosslinked at 2:1 ratio.

Figure 5.6: Contraction of plasma hydrogels in terms of weight loss containing three concentrations of thiolated HA (0.05%, 0.1% and 0.2%) and crosslinked at 6:1 ratio.

Figure 5.7: Contraction of plasma hydrogels in terms of weight loss containing three concentrations of thiolated HA (0.05%, 0.1% and 0.2%) and crosslinked at 10:1 ratio.

Figure 5.8: Contraction of plasma hydrogels in terms of weight loss containing three concentrations of thiolated HA (0.05%, 0.1% and 0.2%) and crosslinked at 14:1 ratio.

by increasing the internal motility of the hydrogel.

On the other hand, hydrogels containing 0.1% of HA show that the total amount of proteins released decrease in roughly 5 mg for no crosslinker and this effect is further accentuated when PEGDA is incorporated although no differences were found among crosslinking levels (Figure 5.9B). Similar behaviour was found for hydrogels with 0.2% of HA, a decrease of 2.5 mg to 5 mg of protein released with no differences this time between the hydrogels crosslinked with PEGDA and the hydrogels only containing thiolated HA (Figure 5.9C).

5.3.4 HA release from hydrogels

The amount of thiolated HA that is able to scape the matrix after 24 hours of incubation in PBS at 37°C was measured through a sandwich ELISA assay kit specific for HA and provided by Echelon Biosciences. Samples with three levels of HA concentration (0.05%, 0.1% and 0.2% w/v) and two levels of PEGDA crosslinking (2:1 and 14:1) were left at 37°C immersed in PBS and aliquots were taken after 24 hours from the surrounding medium and diluted in order to quantify the total amount of HA present.

The results obtained show that the higher degree of crosslinking inhibits the escapement of thiolated HA into the medium at all HA concentrations and thus better maintains the structure of the plasma-derived fibrin hydrogels through time (Figure 5.10). Furthermore, same levels of total μg released to the medium were found regardless of the initial quantity of HA added. Evidencing the independence of the process of release of HA from the plasma hydrogels from the initial amount present in the structure (Figure 5.10).

5.3.5 Mechanical characterisation: compression test

Uniaxial compression test was performed in order to study the changes in elasticity modulus or compression or Young's modulus of the plasma hydrogels when thiolated HA crosslinked with PEGDA is added to the structure. The measurements were performed at an Instron 3366 machine at CSIC and two parameters were evaluated: the compressive modulus of elasticity at 20% strain and the maximum load withstand by the hydrogels at 70% strain.

Figure 5.9: Cumulative protein release from the plasma-derived hydrogels containing three HA concentrations A) 0.05%, B) 0.1% and C) 0.2% and four levels of PEGDA crosslinker (2:1, 6:1, 10:1 and 14:1).

Figure 5.10: Amount of HA (µg) released after 24 hours from the plasma hydrogels containing 0.05%, 0.1% and 0.2% of HA at two different crosslinking ratios (2:1 and 14:1).

The results obtained for the Young's modulus at 20% strain, which belongs to the linear region of the stress vs. strain curve, show the increase in the elasticity of the hydrogels with the increasing amount of thiolated HA added (0.05%, 0.1% and 0.2% w/v)(Figure 5.11). Regarding the level of crosslinking, comparing the maximum level 2:1 to the minimum level 14:1 results obtained show that increasing the crosslinker decreases the overall elasticity of the plasma-derived hydrogels compared to lower levels of crosslinker used. The incorporation of HA at all concentrations (0.05%, 0.1% and 0.2% w/v) and crosslinking levels (2:1 and 14:1) yielded a greater modulus of elasticity compared to the plasma control (Figure 5.11).

Furthermore, the maximum load that the hydrogels were able to endure in the compressive test was calculated at 70% strain yielding as result the increase in load measured in Newtons (N) for all composition with the increasing amount of HA (0.05%, 0.1% and 0.2% w/v) and the increasing level of PEGDA crosslinker (14:1, 10:1, 6:1 and 2:1)(Figure 5.12). The maximum load withstand was measure for the hydrogels containing 0.2% of HA and 2:1 level of crosslinking at 23.3 ± 5.1 N (Figure 5.12A).

Figure 5.11: Mechanical characterisation in terms of compressive modulus of elasticity (Pa) of plasma hydrogels containing 0.05%, 0.1% and 0.2% of HA at two different crosslinking ratios (2:1 and 14:1).

5.3.6 Structural characterisation (SEM)

Plasma hydrogels containing thiolated HA crosslinked with PEGDA were structurally characterised through SEM as reported in previous chapters. In brief, plasma hydrogels containing 0.1% HA and 0.2% HA with 2:1 level of crosslinking were dried with supercritical CO₂ point drying method and visually inspected through scanning electron microscopy to check their differences with plasma control and plasma after 7 days of PBS incubation.

The results show that incorporating both concentrations of HA and using PEGDA as crosslinker agent affects to the internal structure of the plasma-derived hydrogels (Figure 5.13). Specifically, differences in porosity were found between hydrogels containing 0.1% of thiolated HA (Figure 5.13C) and plasma (Figure 5.13A) and an even more compact structure was found when higher

Figure 5.12: Mechanical characterisation in terms of maximum withstood load of plasma hydrogels containing 0.05%, 0.1% and 0.2% of HA crosslinked at ratios A) 2:1; B) 6:1; C) 10:1; D) 14:1.

amounts of HA (0.2%) and crosslinker (2:1) were added to the hydrogels (Figure 5.13D). The changes shown between plasma control at 0 days and 7 days incubation were mainly in the smoothness and size of the fibrin fibres (198 ± 35 nm for the control and 102 ± 23.5 nm for 7 day hydrogels) and the disappearance of platelets in the structure (Figure 5.13A and 5.13B).

5.3.7 Fibroblast-mediated contraction

The capacity of thiolated HA crosslinked with PEGDA to inhibit the contraction produced by human primary fibroblast inside the plasma-derived matrices

Figure 5.13: Scanning electron microscopy images of A) Plasma hydrogel; B) Plasma hydrogel after 7 days incubated in PBS at 37°C; C) Plasma hydrogel containing 0.1% of HA; D) Plasma hydrogel containing 0.2% of HA and 2:1 ratio of PEGDA crosslinking.

was evaluated through a contraction in area study at different time points (0h, 4h, 6h, 8h, 24h, 48h, 72h, 7 days, 10 days and 15 days).

The results show that increasing the amount of thiolated HA inhibits the contraction of the hydrogels after 72 hours 14% for HA 0.05%, 25% for HA 0.1% and 40% for HA 0.2% with respect to the plasma control when using 6:1 ratio of PEGDA crosslinker (Figure 5.14). Moreover, the effect of the different levels of crosslinkage was proven to impact the contractile behaviour by significantly inhibiting the loss of surface area compared to adding the thiolated HA alone (Figure 5.15). This effect was further seen with HA levels of 0.2% in concentration, where crosslinking at 6:1 ratio yielded as result a great level of contraction inhibition with respect to plasma and also other lower levels of crosslinking (Figure 5.16). Figure 5.17 shows representative images of the fibroblast-mediated contraction for plasma and HA 0.2% 6:1 hydrogels up to

15 days incubation in supplemented DMEM at 37°C.

Figure 5.14: Cell-mediated contraction of plasma-derived hydrogels containing 0.05%, 0.1% and 0.2% of HA at 6:1 crosslinking ratio.

5.3.8 Fibroblast proliferation assay

Potential changes in the proliferation of human primary fibroblast inside modified plasma-derived hydrogels containing three concentrations of thiolated HA (0.05%, 0.1% and 0.2% w/v) and four levels of PEGDA crosslinking (2:1, 6:1, 10:1 and 14:1) was monitored through a metabolic activity assay, Alamar BlueTM. The changes in fluorescence produced by the metabolically active fibroblast were recorded at 530 nm excitation wavelength and 590 nm emission wavelength filters with a microplate reader.

The results obtained for the hydrogels containing 0.05% of HA at all crosslinking levels shows an overall increase in proliferation after 7 and 14 days for hydrogels containing no crosslinker and 6:1 and 14:1 PEGDA ratios (Figure 5.18A). Proliferation activity was maintained with 2:1 and 10:1 ratios of crosslinking after 7 days; while at 14 days this last condition (2:1) showed worse performance with respect to plasma control although not statistically significant differences were found (Figure 5.18A).

Figure 5.15: Cell-mediated contraction of plasma-derived hydrogels containing 0.05% of HA at 2:1, 6:1, 10:1 and 14:1 crosslinking ratios in terms of total area contracted in mm².

Figure 5.16: Cell-mediated contraction of plasma-derived hydrogels containing 0.2% of HA at 2:1, 6:1, 10:1 and 14:1 crosslinking ratios.

Figure 5.17: Representative images of the evolution of plasma hydrogel and HA 0.2% 6:1 hydrogel containing embedded fibroblast after incubation in DMEM 10X FBS and 1% P/S at 37°C for time points 0h, 48h, 10 days and 15 days.

Regarding the hydrogels containing 0.1% of thiolated HA, the results obtained yielded a decrease in human primary fibroblast proliferative activity after 7 days of incubation for all hydrogels containing any level of crosslinking ratio (2:1, 6:1, 10:1 and 14:1). However, after 14 days, proliferation activity was recovered to control levels for crosslinking ratios 2:1, 10:1 and 14:1 (Figure 5.18B).

Incorporating the highest concentration of thiolated HA (0.2%) yielded again an overall decrease in the proliferation after 7 days for all the hydrogels containing HA regardless of the crosslinking level or the no use of crosslinker at all (Figure 5.18C). Again, after 14 days of incubation the metabolic activity of fibroblast reached control levels except for the 2:1 PEGDA crosslinking ratio that performed worse probably due to a less porous matrix at the beginning that impeded the correct spreading and proliferation of primary fibroblast embedded in them.

5.3.9 Live/Dead assay

Proliferation and cell viability were further characterised through the Live/Dead assay. This assay was performed to characterize not only the viability and overall proliferation of the embedded fibroblast but to corroborate the non-cytotoxic

Figure 5.18: Fibroblast proliferation inside the plasma hydrogels containing three HA concentrations A) 0.05%, B) 0.1% and C) 0.2% at all levels of crosslinking (2:1, 6:1, 10:1 and 14:1) measured through Alamar Blue™ assay.

nature of the incorporated HA.

The results obtained show that the fibroblasts present a similar morphology and proliferation when 0.1% and 2:1 crosslinking ratio is used compared to the 1.2 mg/ml plasma control (Figure 5.19C). However, when the amount of HA was doubled to 0.2% and 2:1 crosslinking ratio, fibroblast lagged in their spreading inside the matrix and more round shapes were found in the images (Figure 5.19D). A decrease in proliferation was also found for this condition as previously corroborated by the Alamar BlueTM assay. A negative control to check that the Live/Dead kit was working was prepared and the results showed circular-shaped dead cells embedded in the hydrogel.

5.3.10 Keratinocyte proliferation assay

The effect of the incorporation of thiolated HA and PEGDA crosslinker in the attachment and proliferation capacity of primary human keratinocytes was monitored through a MTS assay.

The results obtained show that increasing the amount of thiolated HA decreases the proliferative activity of primary keratinocytes regardless of the level of crosslinking used both after 4 days and 7 days of incubation (Figure 5.20). There was one condition that behaved in a different manner (HA 0.1% 10:1) probably due to an experimental mistake in the number of cells added (Figure 5.20C). Interestingly, hydrogels containing 0.05% at the higher crosslinking ratios (2:1 and 6:1) showed an increase in the proliferative activity of primary fibroblast after 7 days, indicating their increased value for the formation of the epithelium in *in vitro* skin constructs (Figure 5.20A and 5.20B).

5.3.11 Organotypic skin generation with thiol-modified HA crosslinked with PEGDA

In vitro skin generation with plasma-derived hydrogels containing thiolated HA and PEGDA crosslinker was performed in an air-liquid interface for the correct differentiation of the epithelium layer formed by primary keratinocytes. The constructs were left for maturation 18 days before processing the samples by fixing the tissues in formaline. Complete characterisation with Hematoxylin &

Figure 5.19: Fibroblast proliferation inside the plasma hydrogels after 48 hours containing A) Control at 1.2 mg/ml fibrin concentration; B) Negative control with cells killed at 65°C for 5 minutes; C) HA 0.1% 2:1; D) HA 0.2% 2:1. All measured through Live/Dead cytotoxicity assay.

Eosin staining plus a range of antibodies against the main proteins present in the skin were used.

The organotypic constructs showed no visual differences between the plasma control hydrogels and the hydrogels containing 0.05% of HA and 2:1 ratio of PEGDA crosslinking after 4 and 16 days of keratinocyte maturation (Figure 5.21). Constructs containing a higher concentration of HA and crosslinker did not allow keratinocyte differentiation as evidenced in figure 5.22 and supported by MTS data (Figure 5.20).

At the time of stopping the maturation process after 18 days, differences in

Figure 5.20: Keratinocytes proliferation on top of the plasma hydrogels containing three levels of HA concentration (0.05%, 0.1% and 0.2% w/v) and crosslinking at A) 2:1 ratio; B) 6:1 ratio; C) 10:1 ratio; D) 14:1 ratio.

the height of the completely formed *in vitro* skin construct were found. In the case of skin containing HA at 0.05% and 2:1 PEGDA ratio, the difference can be appreciated in figure 5.23.

5.3.12 Histological characterisation

Immunohistochemical characterisation of the skin was performed in order to assess the correct formation of the skin. Detection of filaggrin (Figure 5.25), keratin 5 (Figure 5.26), keratin 10 (Figure 5.27), vimetin (Figure 5.28) and collagen VII (Figure 5.29) was assessed in the samples as well as the skin morphology through H&E staining (Figure 5.24).

Human plasma-derived fibrin hydrogels that formed an organotypic skin were used as control and from figure 5.24 two main parts (epidermis and der-

Figure 5.21: Plasma-derived *in vitro* skins generated in the lab containing A) plasma 1.2 mg/ml at day 4; B) plasma 1.2 mg/ml at day 16; C) HA 0.05% at day 4; D) HA 0.05% at day 16.

Figure 5.22: Plasma-derived *in vitro* constructs generated in the lab containing HA 0.2% 2:1 PEGDA crosslinking ratio showing no differentiation of the keratinocyte layer after 2 and 7 days in air-liquid interface.

Figure 5.23: Plasma-derived *in vitro* skins with different final thickness due to the presence of HA.

mis) can be easily differentiated with the H&E staining. Plasma-derived fibrin hydrogels containing 0.05% of thiol-modified HA and 2:1 ratio of PEGDA crosslinking were used as study subjects for this thesis. From figure 5.24, the skin generated after 18 days *in vitro* containing HA showed a much thicker dermis compared to the control although the fibroblast embedded on them were not as spread as expected.

The expression of common skin markers was positive for the proteins tested in both the control and the hydrogels containing 0.05% HA and 2:1 PEGDA crosslinking ratio, evidencing the correct formation of skin *in vitro* in the presence of thiol-modified HA (Figures 5.25, 5.26, 5.27 and 5.28). Furthermore, better expression was found for keratin 10 for hydrogels containing HA, as it showed a more confined expression in the suprabasal layer of the epidermis, where it is naturally found (Figure 5.27). Morphologically, a good epidermis with stratum corneum was correctly formed as evidenced from the expression of filaggrin, K5 and K10 in the correct positions (Figures 5.25, 5.26 and 5.27). The only skin marker that was found to have an abnormal expression was collagen type VII, compared with the control the deposition of collagen VII did not took place in the organotypic skins but rather stayed inside the basal layer of keratinocytes (Figure 5.29).

Figure 5.24: Plasma-derived *in vitro* skins characterised through H&E staining containing only plasma or plasma with 0.05% HA and 2:1 PEGDA crosslinking ratio.

Figure 5.25: Plasma-derived *in vitro* skins characterised through immunohistochemistry showing expression of filaggrin in hydrogels containing only plasma and plasma with 0.05% HA and 2:1 PEGDA crosslinking ratio.

Figure 5.26: Plasma-derived *in vitro* skins characterised through immunohistochemistry showing expression of keratin 5 in hydrogels containing only plasma and plasma with 0.05% HA and 2:1 PEGDA crosslinking ratio.

Figure 5.27: Plasma-derived *in vitro* skins characterised through immunohistochemistry showing expression of keratin 10 in hydrogels containing only plasma and plasma with 0.05% HA and 2:1 PEGDA crosslinking ratio.

Figure 5.28: Plasma-derived *in vitro* skins characterised through immunohistochemistry showing expression of vimentin in hydrogels containing only plasma and plasma with 0.05% HA and 2:1 PEGDA crosslinking ratio.

Figure 5.29: Plasma-derived *in vitro* skins characterised through immunohistochemistry showing expression of collagen type VII in skin biopsy, hydrogels containing only plasma and plasma with 0.05% HA and 2:1 PEGDA crosslinking ratio.

5.4 Discussion

The incorporation of a new polymer network inside plasma hydrogels consisting of a commercially modified thiolated HA and PEGDA crosslinker was demonstrated in this work. This achievement opens the possibility of incorporating multiple polymer of different nature in order to enhance the performance of dermo-epidermal equivalents derived from human plasma. As explained in the introduction, the crosslinking occurs by means of the simple click chemistry, capable of yielding high efficiency in the reaction with no unreactive species generating side products and under mild conditions compatible with biological processes [10].

In this chapter, the addition of three concentrations of HA (0.05%, 0.1% and 0.2% w/v) with four levels of PEGDA crosslinking (2:1, 6:1, 10:1 and 14:1) have been evaluated through a series of experiments comprising both the material and biological properties of the newly formed hydrogels.

Gelation time was found to be influenced by the incorporation of the new HA-PEGDA matrix (Figure 5.2). For clinical purposes it is desirable for gelation time to take as short time as possible due to potential cases in which the matrices are needed immediately. Furthermore, with the rise of 3D bioprinting technology short gelation times might be of critical importance in the design of new bioinks for their use as skin matrices, needing the gelation to occur shortly after the mixture comes out of the nozzle [11]. In the case explained in this work, although gelation time significantly increased with the incorporation of HA and PEGDA, it did so by adding as much as ten minutes to the overall reaction, meaning no impact in the outcome was recorded due to the 1 hour gelation period that was allowed for all hydrogels.

The reason behind this lag in hydrogel formation when HA and PEGDA were added resides in the disruption of the fibrin network polymerization. HA is able to interact sterically with components present in the human plasma samples provided by the blood bank [12]. This interactions delay the polymerization of the fibrin network, yielding longer times for the hydrogels to form in the presence of HA and PEGDA.

The delay in gelation time was further evidenced by the change in O.D. recorded through UV-Vis spectroscopy, showing both longer times to the start

of the change in turbidity for HA-PEGDA incorporated hydrogels and a lower level of O.D. reached due to the transparent nature of the HA-PEGDA network (Figures 5.3 and 5.4).

Regarding the swelling behaviour of the plasma-derived fibrin hydrogels incorporated with thiolated HA and PEGDA, it was shown that a decrease in the contraction behaviour ranging from a mere 5% to a whopping 25% occurred for all the conditions tested (Figures 5.5, 5.6, 5.7 and 5.8). The study of the mass loss of hydrogels is of vital importance in the clinical application, as an excessive contraction would lead to a failure in the transplant or in the development of scar tissue or even fibrotic scarring [13, 14]. The swelling behaviour is based on the presence of hydrophilic groups in the composition of the hydrogel (-COOH; -SO₃H; -OH; -CONH) [15] and on the environmental conditions such as temperature and pH that can greatly influence the behaviour of hydrogels [16]. In the case of thiolated HA, the presence in its structure of polar groups (-OH; -COOH and -SH) might influence the swelling behaviour by retaining more water inside the structure than the control plasma hydrogels.

The protein release study revealed the necessary information regarding the presence of proteins and their influence in the correct formation of skin *in vitro* and the role of the plasma proteins in wound healing. It is known that the main proteins conforming the human blood plasma, fibrinogen, thrombin and other tissue factors greatly favour the wound healing process by increasing the proliferation of migrating cells and thus speeding the rate of remodelling of the wounded site [17]. Our experiments show a decrease in the amount of proteins released from the hydrogels when both 0.1% and 0.2% of HA is incorporated regardless of the crosslinking level (2:1, 6:1, 10:1 or 14:1), thus indicating a potential advantage with respect to the plasma-derived fibrin hydrogels (Figure 5.9). In the case of hydrogels containing 0.1% HA, increasing the crosslinking ratio further inhibited the release of proteins compared with just adding HA, as expected from results found for other materials [18].

Furthermore and apart from studying the release of proteins from the matrices, the escapement of the incorporated HA from the hydrogels after incubation in PBS was also studied. The result obtained shows that a minimal part (1-10%) of the added HA is able indeed to escape the matrix (Figure 5.10).

Mechanical properties of the scaffolds play a crucial role in the correct development of engineered tissue both *in vitro* and *in vivo*. Skin has been previously characterised mechanically, yielding torsion strength values ranging from 0.4 to 0.85 MPa for the dermal component [19]. A majority of current scaffolds designed to bear cells inside have been reported to have very poor mechanical properties leading to early stage failures in the development of engineered tissues [20]. In this context, the incorporation of thiolated HA crosslinked with PEGDA has demonstrated an increase in stiffness of the hydrogels with respect to the plasma control. The natural stability of the HA-PEGDA hydrogels comes from the bonds formed through thiol-ene chemistry and the different levels of crosslinking, that have been demonstrated to be able to tune the mechanical properties of the hydrogels [21]. The compressive test carried out in this thesis gave us information about the stiffness and elasticity of the matrices by means of the compressive modulus of elasticity or Young's modulus. When HA-PEGDA was incorporated into the plasma-derived fibrin hydrogels, an increase in the Young's modulus evidenced the increase in stiffness for these types of modified gels with the increasing amount of HA concentration (0.05%, 0.1% or 0.2% w/v) (Figure 5.11). Interestingly, at low crosslinking values (14:1), the interactions between the HA and fibrin seem to be prevalent, giving higher levels of stiffness; while at high levels of crosslinking (2:1) the higher elasticity of the commercial HA-PEGDA preparation prevailed.

The internal structure of the hydrogels containing thiolated HA and PEGDA was studied through SEM. The differences found in structure were due to the presence of thiolated HA into the structure, making the hydrogels more compact, with more but smaller pores in the structure (Figure 5.13). Comparatively, plasma hydrogels incubated for 7 days showed smoother fibres than their 0 days counterpart, due to the wash out of proteins and lipids from the hydrogels [17]. In terms of fibres diameters, a decrease was also found between these two conditions, the only ones where clean fibres could be measured. Thicker fibres found in the HA-PEGDA plasma derived hydrogels are a consequence of the interaction between fibrin and HA leading to the thickening of fibres due to the lateral aggregation of the fibrils as previously reported [22].

Continuing with the contraction of the hydrogels, now the effect of human primary fibroblast on the contractile behaviour of gels was investigated. As reported in previous chapters, it was expected that cells were able to contract

the matrices and even completely degrade the hydrogels in the case of fibrin after transplantation as previously reported in the literature [14, 23]. Thus it was of vital importance to completely understand the mechanism by which the cells attach to the hydrogels and are able to contract them.

The results evidence that the increase in rigidity of the matrices when the HA-PEGDA is incorporated results in a decrease in contraction in the presence of primary fibroblast (Figures 5.14, 5.15 and 5.16). Other factor that might be affecting the contraction of the matrices is the correct proliferation and viability of the fibroblast inside the hydrogels containing thiolated HA and PEGDA crosslinker. As shown in the results, an overall decrease in proliferation was found for all hydrogels containing any concentration of HA and level of crosslinker after 7 days with respect to the plasma control (Figure 5.18). This alone is evidencing that fibroblast are finding difficult to spread inside the more compact matrices and remodelling is taking longer than usual [24]. This effect was further evidence by the finding that fibroblasts encapsulated inside the hydrogels at 0.2% HA and 2:1 PEGDA crosslinking were still in a round morphology after 48 hours of incubation in the Live/Dead assay performed (figure 5.19). Fibroblast proliferation activity after 14 days gave us evidence that the type of reaction taking place (thiol-ene chemistry) is suitable for biological components and does not elicit a detrimental reaction for cells [21].

A part from fibroblasts encapsulated inside the matrices, a second type of cellular component is needed for the generation of organotypic skins *in vitro*. Human primary keratinocytes were evaluated in the presence of thiolated HA and PEGDA crosslinker in order to assess their proliferative ability with respect to the plasma-derived matrices used as control. The results in this case show a decrease in the metabolic activity of keratinocytes when they are feeling the more hydrated surface of the matrix when high amounts of HA and PEGDA crosslinker are incorporated (Figure 5.20). This effect caused a decrease in the differentiation rate and thus the incorrect formation of skin tissue in the organotypic skin generation experiment. The more hydrated surface was detrimental as it maintained the surface of the keratinocyte layer with liquid impeding the complete drying of the layer and thus probably inhibiting the correct differentiation into the different strata that conform the epidermis.

The histology results showed that the incorporation of a mild amount of HA

(0.05%) crosslinked at the highest ratio (2:1) was the optimal condition to generate fully differentiated skins while enhancing the mechanical properties of the constructs. Expression of the most common markers of skin (Vimentin, K5, K10 and Filaggrin) was found in the dermo-epidermal equivalents generated with HA crosslinked with PEGDA (Figures 5.25, 5.26, 5.27 and 5.28). Compared to the plasma controls, K10 showed a more confined and homogeneous expression in the suprabasal layer of the epidermis, showing thus a better final skin. Furthermore, dermis was shown to be up to 3 times thicker compared to the plasma control and the formation of a stratum corneum was enhanced for the constructs containing HA crosslinked with PEGDA as evidenced in the filaggrin expression. Collagen type VII was also evaluated although the results were not as good as expected. It seemed that collagen VII had been expressed in the organotypic constructs inside the basal layer of keratinocytes (Figure 5.29). This can be explained knowing that collagen VII expression can occur at the last stages of differentiation, while the constructs were left differentiating for 18 days, less amount of time than the normal 21 days in culture. However, Cubo et al. reports the correct formation of the dermo-epidermal membrane evidenced through collagen VII in immunofluorescence technique after just 17 days of differentiation *in vitro* [25]. Thus it would be optimal to either optimize the immunohistochemical protocol due to problems with proteinase K incubation time and samples destruction or try with the frozen samples and check the presence of collagen VII through the immunofluorescence technique.

In this sense, after the complete evaluation of the results obtained the condition selected to perform the *in vivo* experiment was carefully determined, as it comprised the use of animals, in this case mice, to confirm our hypothesis. A mild amount of thiolated HA (0.05%) and the highest degree of crosslinking (2:1) was the final condition utilised for the nude mice back transplantation with human organotypic skin constructs explained in chapter 7.

Bibliography

- [1] B. Ruszymah, K. Chua, A. Mazlyzam, and B. Aminuddin, "Formation of tissue engineered composite construct of cartilage and skin using high density polyethylene as inner scaffold in the shape of human helix," *International journal of pediatric otorhinolaryngology*, vol. 75, no. 6, pp. 805–810, 2011.
- [2] R. J. Faust, "Technical Manual of the American Association of Blood Banks," *Anesthesiology*, vol. 65, no. 2, p. 242, 1986.
- [3] E. A. BIO - Division of BioTime, "HyStem ® Hydrogels Cellular Matrices for Translational Research," tech. rep.
- [4] M. K. Cowman, H.-G. Lee, K. L. Schwertfeger, J. B. McCarthy, and E. A. Turley, "The content and size of hyaluronan in biological fluids and tissues," *Frontiers in immunology*, vol. 6, p. 261, 2015.
- [5] A. Meana, J. Iglesias, M. Del Rio, F. Larcher, B. Madrigal, M. Fresno, C. Martin, F. San Roman, and F. Tevar, "Large surface of cultured human epithelium obtained on a dermal matrix based on live fibroblast-containing fibrin gels," *Burns*, vol. 24, no. 7, pp. 621–630, 1998.
- [6] S. G. Llames, M. Del Rio, F. Larcher, E. García, M. García, M. J. Escamez, J. L. Jorcano, P. Holguín, and A. Meana, "Human plasma as a dermal scaffold for the generation of a completely autologous bioengineered skin," *Transplantation*, vol. 77, no. 3, pp. 350–355, 2004.
- [7] P.-H. D. Edqvist, L. Fagerberg, B. M. Hallstrom, A. Danielsson, K. Edlund, M. Uhlen, and F. Ponten, "Expression of human skin-specific genes defined by transcriptomics and antibody-based profiling,," *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society*, vol. 63, pp. 129–141, feb 2015.
- [8] M. Albanna and J. H. Holmes IV, *Skin tissue engineering and regenerative medicine*. Academic Press, 2016.
- [9] Student, "The probable error of a mean," *Biometrika*, pp. 1–25, 1908.
- [10] H. C. Kolb, M. Finn, and K. B. Sharpless, "Click chemistry: diverse chemical function from a few good reactions," *Angewandte Chemie International Edition*, vol. 40, no. 11, pp. 2004–2021, 2001.

- [11] S. V. Murphy and A. Atala, “3d bioprinting of tissues and organs,” *Nature biotechnology*, vol. 32, no. 8, p. 773, 2014.
- [12] J. R. E. Fraser, T. C. Laurent, and U. Laurent, “Hyaluronan: its nature, distribution, functions and turnover,” *Journal of internal medicine*, vol. 242, no. 1, pp. 27–33, 1997.
- [13] S. V. Murphy, A. Skardal, and A. Atala, “Evaluation of hydrogels for bioprinting applications,” *Journal of Biomedical Materials Research Part A*, vol. 101, no. 1, pp. 272–284, 2013.
- [14] F. Lee and M. Kurisawa, “Formation and stability of interpenetrating polymer network hydrogels consisting of fibrin and hyaluronic acid for tissue engineering,” *Acta Biomaterialia*, vol. 9, pp. 5143–5152, feb 2013.
- [15] A. Patel and K. Mequanint, “Hydrogel Biomaterials,” in *Biomedical Engineering - Frontiers and Challenges*, InTech, aug 2011.
- [16] D. Velasco, C. Elvira, and J. San Román, “New stimuli-responsive polymers derived from morpholine and pyrrolidine,” *Journal of Materials Science: Materials in Medicine*, vol. 19, no. 4, pp. 1453–1458, 2008.
- [17] R. Corrales, M. Sikorski, M. García, D. Velasco, and J. Jorcano, “Mechanical modulation of a human plasma based skin scaffold via reactive multi-arm polyethylene glycols,” *Biomecánica*, jul 2018.
- [18] C.-C. Lin and K. S. Anseth, “Peg hydrogels for the controlled release of biomolecules in regenerative medicine,” *Pharmaceutical research*, vol. 26, no. 3, pp. 631–643, 2009.
- [19] C. Pailier-Mattei, S. Bec, and H. Zahouani, “In vivo measurements of the elastic mechanical properties of human skin by indentation tests,” *Medical engineering & physics*, vol. 30, no. 5, pp. 599–606, 2008.
- [20] F. J. O’Brien, “Biomaterials & scaffolds for tissue engineering,” *Materials Today*, vol. 14, pp. 88–95, mar 2011.
- [21] G. D. Prestwich, “Hyaluronic acid-based clinical biomaterials derived for cell and molecule delivery in regenerative medicine,” *Journal of controlled release*, vol. 155, no. 2, pp. 193–199, 2011.

- [22] R. D. LeBoeuf, R. R. Gregg, P. H. Weigel, and G. M. Fuller, “Effects of hyaluronic acid and other glycosaminoglycans on fibrin polymer formation,” *Biochemistry*, vol. 26, no. 19, pp. 6052–6057, 1987.
- [23] M. Ahearne, “Introduction to cell–hydrogel mechanosensing,” *Interface focus*, vol. 4, no. 2, p. 20130038, 2014.
- [24] L. Almany and D. Seliktar, “Biosynthetic hydrogel scaffolds made from fibrinogen and polyethylene glycol for 3d cell cultures,” *Biomaterials*, vol. 26, no. 15, pp. 2467–2477, 2005.
- [25] N. Cubo, M. Garcia, J. F. Del Canizo, D. Velasco, and J. L. Jorcano, “3D bioprinting of functional human skin: production and in vivo analysis,” *Biofabrication*, vol. 9, p. 15006, dec 2016.

Conclusions

This thesis has presented a thorough study of the use of human plasma-derived hydrogels and their enhancement through the incorporation of thiol-modified HA crosslinked with PEGDA for the treatment of skin wounds in the form of extensive burns, chronic ulcers and other acute deep wounds. It started by investigating the nature of the skin substitutes patented by this group and its use in transplantation of large-surface burnt patients in Spain. Plasma was being used at different concentrations before this research effort tried to shine light on the optimal conditions to generate the best dermo-epidermal skin substitutes to maximize their positive outcomes both *in vitro* and in the clinic. Several issues have always accompanied the hydrogels based on the polymerization of fibrin to generate hydrogels appropriate for aiding in skin damage repair. One of these persisting issues has been the poor mechanical properties reported for these systems, with elastic moduli in the range of 10 Pa. With such poor stability, implementation of these hydrogels has not reached its full potential. The fact that plasma is present on the natural response of the body to wounds makes these fibrin based matrices the perfect candidate to enhance the wound healing process and even try to completely regenerate the tissue. In that way, the main objective set for this thesis was the optimization and the enhancement of the plasma-derived matrices in terms of mechanical performance and biological properties for their implementation in patients with overall better outcomes and healing times. The second objective proposed was the creation of a better *in vitro* skin model to serve as testing platform for topical drugs and cosmetics and that could render the animal testing obsolete. In order to achieve the goals stated, the thesis was divided into four different studies involving first the characterisation of the material and the hydrogels and later the generation of an improved matrix capable of forming a mature skin *in vitro* and been capable to be transplanted and functional *in vivo*.

The second chapter of this thesis has described the difference in performance *in vitro* found for two different final plasma concentrations (1.2 mg/ml and 2.4 mg/ml). The research performed in this topic led us to determine the suitability of hydrogels at lower fibrin concentration for the generation of dermo-epidermal equivalents due to the better performance of cells inside a less compacted matrix. Furthermore, due to the limitations in material quantity since there is a limited amount of plasma that can be obtained either from donors or the patients, the selection of 1.2 mg/ml of fibrin concentration for the remaining of this thesis was in accordance with the maximum optimization to generate large skin surfaces for patient transplantation.

The third chapter centred its attention on the early shrinkage of these matrices *in vitro* and the main driver of this behaviour was elucidated. Excessive contraction found in the fibrin-based matrices both in transportation to the clinic and when generating organotypic skin *in vitro* led us to try to unveil the reason behind it. Although common sense would state that cell-mediated contraction of the matrices is the main driver, release of proteins present in plasma and changes in the conformation of fibrin due to the surrounding media were found to also affect and contribute to this contraction effect. This investigation served to optimise the culture conditions and behaviour of the fibrin matrices under different temperatures and to find ways to minimise the contractile behaviour in transport conditions. Although this chapter aims to tackle contraction *in vitro*, poor mechanical properties were still a concern for clinicians in the operating rooms. In that sense, the incorporation of another material able to reinforce the matrix was taken into consideration.

In chapter 4 we present the incorporation of natural, non-modified HA of three levels of MW (500kDa, 700kDa and 1MDa). Hyaluronic acid (HA) is naturally present in the skin and contributes during skin wound healing by helping the re-epithelialization of the tissue. It is also known to have a role in withstanding the mechanical pressure suffered by tissues like skin and cartilage. These two main properties made HA the most suitable material to reinforce our matrix. The results obtained for natural HA were not satisfactory in terms of contraction and mechanical properties, the two main properties to be improved. Contraction of plasma hydrogels was found to be increased with the presence of HA compared to the control, in the same manner, compressive modulus of elasticity and maximum load withstand were reported to be lower than the

controls. Natural HA is known to only interact with fibrin through reversible ionic bonds which was the main reason behind the poor results obtained. The solution proposed came in the form of a synthetically modified HA that could form a parallel crosslinked network that could better reinforce the matrix and avoid contraction while maintaining or improving the biological properties.

Chapter 5 is devoted to the incorporation of a thiol-modified HA capable of forming and interpenetrating polymer network (IPN) with the fibrin matrix and thus enhance its performance in terms of contraction, mechanical properties and biological response. Thiolated HA crosslinked with PEGDA was successfully incorporated into the human plasma-derived hydrogels and the contractile behaviour, mechanical properties and other material characteristics were studied. This new material was proven to reduce the level of contraction *in vitro* and confer a higher mechanical performance in terms of elastic modulus and load withstand. Furthermore, low levels of HA (0.05%) and the highest level of crosslinking (2:1) resulted in the optimal condition for improved cell behaviour. Both fibroblast proliferation inside the matrix and keratinocyte attachment and proliferation when seeded on top of the matrices to form the epidermis were found to improve. With this condition, *in vitro* skins were generated yielding improved K10 expression and better stratum corneum formation. Skins were also found to have a thicker dermis, evidencing the reduction in contraction in the z-axis conferred by the presence of thiolated HA in the structure.

This thesis has served as proof that the optimisation of human plasma-derived hydrogels for skin tissue engineering is achievable and that there is always room for improvements to be found in the form of new combination of materials that can better mimic the *in vivo* conditions found in nature. Better resemblance of these tissues will lead to cells behaving in a more similar manner as they do in their natural environment, opening the possibility to explore factors and cell types that completely regenerate tissues.

Future work

The validation of the correct function of modified plasma-derived hydrogels containing thiolated HA and PEGDA crosslinker was made through the implantation of the hydrogels into nude-immunodeficient mice. In order to do so, hydrogels containing 0.05% of thiolated HA and 2:1 PEGDA crosslinking ratio were prepared containing 60,000 fibroblasts/ml of hydrogels at a final volume of 3.5 ml in 6-well plates. Two plasma control hydrogels were also prepared in order to compare the outcome of the skin maturation *in vivo*. After 24 hours of incubation with supplemented medium in order to wash out all the unreacted calcium left in the hydrogels, human primary keratinocytes were seeded on top of the constructs at a final amount of 1,000,000 cells per hydrogel. Primary keratinocytes were left to attach into the matrices for 48 hours before taking them into the animal facility where transplantation would take place.

Transplantation of the hydrogels containing HA into the backs of nude-immunodeficient mice took place at the animal facility in Ciemat. Five mice were intraperitoneally injected with ketamine, an anaesthetic and sedative agent before the start of the procedure. The skin of the back of the mice was then sectioned in a circular form as shown in Figure 7.1 and the skin cut was frozen and defrosted three times with liquid nitrogen in order to devitalize the tissue and use it later as wound dressing for the mice. Open wounds were maintained with a PBS humidified muslin while the freezing cycle was performed. Hydrogels were then put on top of the open wounds and the devitalised skins removed from the back of the mice were sewed again as can be seen in figure 7.1-8. Once the sewing process was finalised, mice woke up after the administration of Atipamizol, a reverser of the anaesthesia.

Mice were carefully put back into labelled boxes and left for 5 weeks to check if the hydrogels constructs have been integrated into the mice. Furthermore,

Figure 7.1: *In vivo* assay procedure 1) NUDE MRI mice; 2) Anesthetized mice; 3) Back of mice disinfected; 4) Skin removal; 5) Hydrogel detachment from plate; 6) Hydrogel held with tweezers; 7) Hydrogel place in the back of the mouse; 8) Devitalized skin used as biological bandage; 9) Surgically closed wound.

9 weeks were needed for the complete maturation of the skin, after which a complete characterisation of the *in vivo* generated skin was performed following the procedure explained in section 5.2.15 of Materials & Methods Chapter 5. First, biopsies from the newly formed skin on the backs of the mice were obtained through 3mm diameter punches that were analysed immunohistologically to discern their human origin. Later, mice were left to recover from the biopsies and allow for wound healing, which was characterised by stopping the process at days 3, 7 and 14 after the injury. Big, 8mm diameter biopsies were then taken from the mice and further analysed to assess the re-epithelialization of the human skin *in vivo*.

In this ongoing experiment, five immunodeficient mice were transplanted with the plasma hydrogels (2 control and 3 HA 0.05% 2:1) and the uptake and maturation of the human skin was assessed after 9 weeks. Mice were visually inspected after 40 days in order to determine the uptake of the transplant on the back of the mice (Figure 7.2). This first results indicated that from the colour of the newly formed skin, the mice had uptaken the transplant. Next step was the full characterisation of the transplants by tissue sectioning and immunohistochemistry after 9 weeks of maturation *in vivo*. Mice were visually inspected again after the 9 weeks to check for the colour and thickness of the skin (Figure 7.3).

First step in the characterisation was performing a small (3mm diameter) punch biopsy on all mice in order to analyse the origin of the skin (Figure 7.4). Mice were then left to re-epithelialize the damaged skin and their progress was stopped at days 3, 7 and 14 of wound healing process. After the re-epithelialization of the injuries, 8mm diameter punch biopsies were taken from the mice and the skin processed immunohistochemically (Figure 7.5)

This first set of mice was found to not have integrated the dermo-epidermal constructs in their backs. Immunohistochemical analysis revealed the absence of human proteins in the regenerated skins. Therefore this experiments will be repeated in the upcoming months when new mice are available for transplantation.

Figure 7.2: NUDE MRI mice 40 days after transplantation with plasma hydrogels and plasma hydrogels containing 0.05% and 2:1 ratio of PEGDA crosslinker.

Figure 7.3: NUDE MRI mice 9 weeks after transplantation with plasma hydrogels and plasma hydrogels containing 0.05% and 2:1 ratio of PEGDA crosslinker.

Figure 7.4: Mice with 3mm diameter punch biopsies in the back.

Figure 7.5: Mice with 8mm diameter punch biopsy in the back after 14 days of re-epithelization with transplant containing 0.05% of HA and 2:1 PEGDA crosslinking ratio.