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SARA CATARINA NUNES DA SILVA SANTOS

Development of injectable hydrogels derived from human platelet lysates

Desenvolvimento de hidrogéis injectáveis a partir de lisados de plaquetas humanas

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica da Doutora Catarina de Almeida Custódio, Investigadora Pós-Doutorada do Departamento de Química da Universidade de Aveiro e do Professor Doutor João Filipe Colardelle da Luz Mano, Professor Catedrático do Departamento de Química da Universidade de Aveiro.

Aos meus pais e à minha avó

o júri

presidente	Prof. Doutora Maria do Rosário Gonçalves dos Reis Marques Domingues professora associada com agregação do Departamento de Química da Universidade de Aveiro
vogal – arguente principal	Doutora Cláudia Alexandra Martins Lobato da Silva professora auxiliar do Instituto Superior Técnico de Lisboa
vogal - orientador	Prof. Doutor João Filipe Colardelle da Luz Mano professor catedrático do Departamento de Química da Universidade de Aveiro

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palavras-chave

Medicina regenerativa, Engenharia de tecidos, hidrogéis, lisados de plaquetas, plasma rico em plaquetas, medicina personalizada

resumo

A medicina regenerativa e a engenharia de tecidos surgiram como alternativas para as terapias atualmente usadas no tratamento e substituição de tecidos ou órgãos lesados. Estas novas terapias combinam o uso de materiais sintéticos ou biológicos ou uma combinação de ambos por forma a produzir um tecido com a mesma estrutura e funcionalidade do original, contribuindo para o tratamento de tecidos e órgãos lesados. No tecido nativo, as células estão rodeadas por uma matriz extracelular que fornece suporte mecânico e biológico às mesmas. Hidrogéis são redes poliméricas com elevado conteúdo de água que podem ser injetados de uma maneira pouco invasiva, reticulando *in situ*. As suas propriedades mecânicas, físico-químicas e biológicas podem ser ajustadas e controladas, fazendo com que estes se tornem fortes candidatos a simular a matriz extracelular encontrada *in vivo* e a fornecer suporte para o crescimento celular. O papel do plasma rico em plaquetas (PRP) e dos lisados de plaquetas humanas (PL) na fabricação de hidrogéis e também como fonte de fatores de crescimento já se encontra descrito. Contudo, os hidrogéis até agora descritos apresentam fracas propriedades mecânicas, são degradados rapidamente e têm um elevado grau de contração. Assim sendo, o principal objetivo deste projeto é a fabricação de um hidrogel a partir de PL, com propriedades mecânicas aumentadas e adaptáveis, estáveis *in vitro* e *in vivo*.

PL metacrilatados (PLMA) resultaram da modificação dos PL com anidrido metacrílico. Quando expostos à radiação ultravioleta os PLMA são capazes de formar hidrogéis por polimerização radicalar. Foram obtidos dois diferentes graus de modificação: PLMA100 (baixo grau de modificação) e PLMA300 (elevado grau de modificação).

A caracterização mecânica dos hidrogéis mostrou que estes têm propriedades mecânicas aumentadas, influenciadas pelo grau de modificação e pela concentração de PLMA. Imagens obtidas por microscopia eletrónica de varrimento (SEM) demonstraram que o grau de modificação e a concentração de PLMA influenciam a porosidade do hidrogel. Baixas concentrações de PLMA apresentam poros mais largos do que elevadas concentrações e ainda o PLMA300 apresenta uma porosidade bastante apertada. Também foram realizados ensaios de libertação de proteína os quais demonstraram uma libertação sustentada de proteína por parte do hidrogel.

Ensaios de cultura celular *in vitro* foram realizados por forma a avaliar o desempenho biológico dos hidrogéis. Células L929 e células estaminais adiposas humanas (hASCs) foram encapsuladas no hidrogel e os resultados demonstram que a viabilidade celular é mantida durante pelo menos 7 dias. Os resultados obtidos para a quantificação de DNA também demonstram que estes hidrogéis suportam a proliferação celular.

Com este trabalho foi possível obter hidrogéis com um elevado conteúdo proteico que podem ser usados como plataforma para cultura celular. Na era atual, tendo em conta a medicina personalizada e a terapia direcionada, estes materiais autólogos poderão ser aplicados na biomedicina, nomeadamente na engenharia de tecidos, reduzindo problemas como rejeição de tecidos, resposta imune e também problemas éticos.

keywords

Regenerative medicine, tissue engineering, hydrogels, platelet lysates, platelet rich plasma, personalized medicine

abstract

Regenerative medicine and tissue engineering (TE) are emerging as alternatives for the currently used therapies to heal or replace injured tissues and organs. TE therapies use synthetic or biological materials or a combination of both in order to produce a tissue with the same structure and functionality as the original one and this way contribute to tissue healing. *In vivo*, cells are surrounded by an extracellular matrix (ECM) that provides mechanical and biological support for cells. Hydrogels are soft and water-rich polymeric networks that can be injected in a minimal invasive manner and reticulate *in situ*. Their mechanical, physiochemical and biological properties can be easily tuned, which makes them ideal candidates to mimic the ECM and support cell growth. The role of platelet rich plasma (PRP) and human platelet lysates (PL) to fabricate hydrogels and as a source of growth factors (GFs) has been previously reported. However, the materials so far reported, suffer from weak mechanical properties, rapid biodegradation and high degree of contraction. Therefore, the main goal of this project was the fabrication of a 3D PL hydrogel, with increased and tunable mechanical properties and *in vitro* and *in vivo* stability.

Methacryloyl platelet lysates (PLMA) were achieved by modification of PL with methacrylic anhydride (MA). PLMA when exposed to UV irradiation are capable of forming hydrogels by radicalar polymerization. Two different modifications were made: PLMA100 (low-modification degree) and PLMA300 (high-modification degree).

Mechanical characterization demonstrate that PLMA hydrogels have increased mechanical properties, influenced by PLMA modification degree and also by the concentration. Scanning electron microscopy (SEM) images demonstrated the influence of modification degree and PLMA concentration in porous network. Lower concentration of PLMA showed large porous than higher concentrations and PLMA300 showed really tight porous. PLMA protein release assays were also performed, demonstrating an overall sustained protein release from the hydrogel.

In vitro cell culture assays were performed in order to evaluate biological performance of PLMA hydrogel. L929 cells and human adipose-derived stem cell (hASCs) were encapsulated within the hydrogels and for both cell types results showed that cell viability is maintained for at least 7 days. DNA quantification demonstrated that produced hydrogels support cell proliferation.

In this work we were capable to obtain hydrogels from a human source with high protein content, which can be used as a platform for cell culture. In the current era, striving toward personalized medicine and targeted therapy, these type of autologous materials will certainly find applications in biomedical applications, namely tissue engineering strategies, reducing problems like tissue rejection, immune response and also reduces ethical and regulatory issues.

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#

2D: two-dimensional

3D: three-dimensional

C

CTGF: connective tissue growth factor

D

DMSO: dimethyl sulfoxide

E

ECGF: epithelial cell growth factor

ECM: extracellular matrix

EGF: epidermal growth factor

EMA: European Medicines Agency

F

FBS: fetal bovine serum

FDA: Food and Drug Administration

FGF: fibroblast growth factor

G

GFs: growth factors

GMP: Good Manufacturing Practice

H

hASCs: human adipose-derived stem cells

HGF: hepatocyte growth factor

I

IGF: insulin-like growth factor

IL-8: interleukin 8

K

KGF: keratinocytes growth factor

M

MA: methacrylic anhydride

P

PDGF: platelet-derived growth factor

PDMS: polydimethylsiloxane

PF4: platelet factor 4

PL: human platelet lysates

PLMA: methacryloyl platelet lysates

PLMA100: PLMA low modification degree

PLMA300: PLMA high modification degree

PPP: platelet-poor plasma

PRP: platelet-rich plasma

R

RBC: red blood cells

S

SEM: scanning electron microscopy

T

TE: tissue engineering

TGF-β: transforming growth factor β

U

UV: ultraviolet

V

VEGF: vascular endothelial growth factor

Chapter I

Background

1. Background

Abstract

Tissue engineering (TE) is one of the most exciting interdisciplinary and multidisciplinary research areas and is growing exponentially over time. Although considerable progress has been made, the development of a biomaterial that recapitulate the complex human biological microenvironment found *in vivo* is still a challenge. Also, there is an increasing interest in the use of autologous approaches in TE strategies.

Recognizing the unprecedented potential of the platelet-rich plasma (PRP)/human platelet lysates (PL) based materials to support three-dimensional (3D) cell culture and considering their actual limitations, such as poor mechanical stability this project aims to develop a robust PRP/PL based hydrogel for applications in TE. This will be made by the chemical modification of proteins present in the PRP/PL to enhance its chemical reactivity towards a range of reactive monomers and the method to prepare de PRP derived 3D matrix. Benefits will come from the possibility of modelling individualized therapies, the use of autologous approaches is highly required, as it avoids the problem of tissue rejection, immune response and also reduces ethical and regulatory issues. Platelets are unique blood elements involved in healing processes and play a considerable role in tissue regenerative processes. Therefore, the potential of human PL based materials is huge.

1.1. Tissue engineering and Regenerative medicine

TE is one of the major areas of regenerative medicine. It is defined as an interdisciplinary field that encompasses biological science and engineering areas applied to the principles of cell transplantation, materials science, and engineering towards the development of biological substitutes that can restore and maintain normal tissue function[1,2]. Human body has the capacity to remodel and regenerate small defects, however, when severe injuries or large defects occur it is harder or even impossible to heal on its own, thus the importance of TE and regenerative medicine[3]. Currently, therapies based on organ and tissue transplantation are widely used as a solution to heal or replace injured tissues and organs but there are still many limitations in this strategy, such as lack of donors, which leads to patient death while waiting for donor organs, and severe immune complications. Considering that, TE may be the answer to bypass these obstacles[1,4].

TE therapies involve numerous strategies like the use of synthetic and biological materials, cells and the combination of both to replace structurally and functionally the missing tissue or to contribute to tissue healing[1]. These TE strategies are usually divided into categories[4,5]:

- direct injection of cells into the tissue of interest or the systematic circulation
- cells implantation after they have been combined to form a 3D tissue structure
- scaffold-based delivery of molecules such as drugs, proteins and oligonucleotides.
- direct delivery of molecules.

TE scaffolds can be synthetic or natural based. Synthetic scaffolds are widely used in TE applications, as the material mechanical properties can be precisely controlled. On the other hand, natural based materials can provide a large variety of biofunctional motifs that regulate cell adhesion and proliferation processes and cell phenotype. So, the ideal scaffold must combine the easily tunable mechanical properties of the synthetic materials, provide biofunctionality of natural based materials[6] and must also facilitate effective nutrient transfer, gas exchange and metabolic waste removal[7].

Extracellular matrix (ECM) is a dynamic structure which provides not only essential physical support for the cellular constituents but also contributes to biochemical signaling, required for tissue morphogenesis, differentiation and homeostasis. ECM is composed of glycosaminoglycans and fibrous proteins like collagen, elastin, fibronectin and laminin and is constantly remodeled by cells during development, homeostasis and wound healing[3,7,8]. Therefore, for TE applications, design and engineer a culture microenvironment that can mimic the native tissue and its ECM, its soluble bioactive factors and products of cell-cell interactions is desirable so they can replicate tissue functions *in vitro*[3,7]. However this is a huge challenge due to the ECM heterogeneity and complexity[7].

Over the past two decades there has been an emergence of the TE industry. Food and Drug Administration (FDA) and European Medicines Agency (EMA) are the responsible entities to approve all the products involved in these areas. However, this is a difficult process because it is time consuming and requires high monetary investment. Approval of drugs or biological products requires many years of development and testing, involving several phases. The efficacy of regenerative medicine and TE products has been increasing and a significant number of products and therapies have received FDA approval and are now

commercially available. For example, the first FDA-approved biologic product for orthopedic applications uses autologous chondrocytes which are harvested, expanded *ex vivo* and implanted in the patient at the site of injury[1].

A reasonable number of conditions have to be taken into account for the advancement and effective clinical application of regenerative medicine and TE therapies. First, isolated stem cells often require the creation of modulated microenvironments that provide specific cues for a tight control over cell behavior, which will lead to efficacy after transplantation and increase their safety profile. Second, most engineered tissues need vascularization for survival and function and thirdly, creating a pro-regeneration environment within the patient may dramatically improve outcomes of regenerative medicine strategies in general[1].

1.2. Cell culture platforms

Culturing cells and tissues *in vitro* in two-dimensional (2D) substrates is still the most used platform for *in vitro* cell culture, however 2D substrates can't mimic cell growth *in vivo*. In 2D cultures, only a segment of the cell membrane can interact with the ECM and neighboring cells (Figure I.1A), while the rest of the cell is exposed to the culture medium. This affect the intracellular signaling, phenotypic fate and interactions with soluble factors[8]. Abnormal cell morphology characteristic of 2D cell culture influences many cellular processes like cell proliferation and differentiation, apoptosis and gene and protein expression[9]. Cells also experience the homogeneous concentration of nutrients, growth factors (GFs) and cytokines present in the culture medium with the section of membrane that contacts the surrounding medium, while *in vivo* the entire cell membrane is in contact with the medium[8]. 2D cell culture limits cells to a planar environment and restricts the more complex morphologies observed *in vivo* and, as a result, 2D substrates cultured cells will not reflect the situation *in vivo*, where cells grow within a complex 3D microenvironment[8,9]. 3D cell cultures platforms have been introduced in order to better mimic mechanical and biochemical signals present in native ECM, providing more physiologically relevant information and more predictive data for *in vivo* tests (Figure I.1B-D)[7]. Table I.1 shows a comparison of cell/tissue behavior under 2D and 3D cell culture conditions.

Recently, an effort has been made in order to develop a variety of 3D cell culture platforms, to be applied in drug discovery, cancer cell biology, stem cell study, engineered functional tissues for implantation and other cell-based analysis[9].

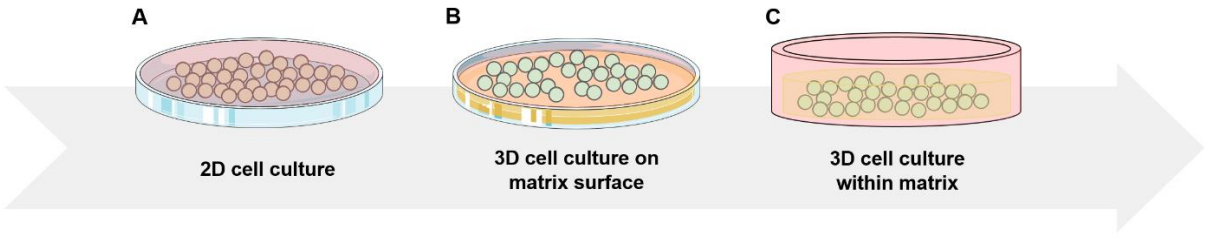

Figure I.1. Schematic representation of a 2D cell culture model (A) and typical 3D cell culture systems: cell growing on matrix surface in contact with culture media (B) and cells embedded within the matrix (C) and submerged in culture media.

Over the past few decades, researchers have explored new materials for cell culture within a 3D matrix, overcoming some of the limitations posed by 2D cell culture systems. 3D cell culture strategies include the combined use of cells and 3D porous scaffold materials serving as structural support for cells. To this end, cells have to be seeded or encapsulated in the 3D matrix. However, there are still some challenges associated with the use of 3D scaffolds. First, the oxygen diffusion within the scaffold requires special notice. Also, regulation of nutrients distribution and metabolites diffusion is an important issue because cellular differentiation and tissue homeostasis will be influenced by its availability. Finally, considering *in vitro* assays, working with 3D networks, cells have limited accessibility for immunostaining, also it may difficult access and extract secreted proteins, DNA or RNA from the cells[8].

Feature/Function	2D culture	3D culture
Tissue-specific architecture	Poor	Rich
Cell morphology	Flat, extended	Round, contracted
Interactions	Limited	Multiple
Cell motility	Fast, free	Slow, restricted
Cell adhesion	Weak	Strong
Cell growth	Directional	All directions
Cell proliferation	High	Low
Apoptosis	Induced	Tissue-like
Intracellular stiffness	Higher in 3D	
Cell polarization	Partly	Full
Extracellular matrix remodeling	Absent or poor	Present
Fluid perfusion	1D	3D
Signaling and diffusion	Asymmetric	Nearly symmetric
Metabolic rate	High	Low

Table I.1. Comparison of cell/tissue behavior under 2D/3D culture conditions (adapted from H. Geckil *et al*, 2010).

In this change from de 2D cell cultures to the 3D cell cultures, hydrogel-based approaches have been widely explored for several biomedical applications[10]. Great efforts have been made in order to demonstrate the usefulness and potential of hydrogels as scaffolds for TE and as drug carriers[3]. In fact, the 3D culture approach is likely to result in many commercial success stories for the key players and several stakeholders in the foreseen future.

1.3. Hydrogels in Tissue Engineering and Regenerative Medicine

The interest in hydrogels for biomedical applications started around 1990s, partly because of emergence of the TE field. Since discovery of hydrogels as a good way to mimic native extracellular matrix (ECM), they have been the target of many studies[7]. Hydrogels are soft 3D crosslinked insoluble hydrophilic network systems with high water content, that allow for an easy transport of oxygen, nutrients and cell metabolites. Hydrogels have received particular attention because of their structural and compositional similarities with ECM and extensive framework for cellular proliferation and survival[7,8,11,12].

In vitro applications of hydrogels are based on the idea that they can work as model systems for: a) drug delivery – the porous structure of hydrogel enables the introduction of a relatively large load of drugs and drug release kinetics can be controlled by controlling hydrogel mechanical, physical and chemical properties; b) diagnostics – hydrogels could be utilized as components of integrated sensors within microdevices providing a better understanding of disease and organ failure caused by agents such as chemical toxins, bacterial and viral pathogens by cell-based chem-bio sensing; c) cell encapsulation and cell culture – the similarities to the ECM composition has proven to be a valuable tool to provide a structure inside where cells can spread and proliferate[7].

In particular, hydrogels designed for cell encapsulation have to be capable of[13]:

- suffer gelation without damaging cells;
- be nontoxic to the cells and surrounding tissues after gelation;
- provide appropriate diffusion of nutrients and metabolites to and from the encapsulated cells;
- have sufficient mechanical integrity and strength to resist to *in vitro* and in *in vivo* implantation;
- *in vivo* degradation with non-toxic degradation by-products.

Variations on the stiffness of the hydrogel are related with changes of the cell morphology, metabolic activity and proliferations rate[12]. Physicochemical properties of the hydrogels depends on the monomers and macromers used, synthesis and fabrication methods, solvent conditions, degradation and mechanical loading history[11,14]. The major physicochemical parameters that determined the properties of a hydrogel are resumed in figure I.2. Adequate mechanical performance of hydrogels depends on specifying, characterizing and controlling mechanical properties of the material like elasticity, viscoelastic behavior, tensile strength and failure strain. These properties are not only affected by polymer and crosslinker characteristics, but also by gelation conditions, gel swelling and degradation[13]. Swelling properties of hydrogels are also important in many applications because it affects some parameters like solute diffusion, surface and mechanical properties and surface mobility. The pore size of the network and interaction between the polymer and the solvent are characteristics that influence the degree of swelling[15].

It is also important to understand and control the degradation rate of hydrogels. There are three mechanisms by which hydrogel degradation occurs: hydrolysis, enzymatic cleavage and dissolution. Hydrolysis occurs normally in synthetic hydrogels at a constant rate *in vivo* and *in vitro* and can be manipulated by the composition of the material. Enzymatic degradation occurs depending on the cleavage sites in the polymer and the amount of available enzymes in the scaffold environment. The dissolution process of degradation depends on the ionic environment in which the ionically crosslinked scaffold is placed[13].

Hydrogels can be classified into natural, synthetic and semi-synthetic according to their source. Synthetic hydrogels can be formed from poly(ethyleneglycol), poly(lactic acid), poly(glycolic acid), poly(lactic-glycolic acid), poly(vinyl alcohol), poly(2-hydroxyethyl methacrylate) among other. Natural based polymers include agarose, alginate, chitosan, hyaluronan, fibrin, collagen, chondroitin sulfate[7,11], laminarin[16], gelatin[17] or matrigel among others. Recently, engineered hybrid hydrogels (combining both types of polymers) have been increasingly reported[11]. In case of using synthetic polymers, they are generally designed to be nontoxic to the cells and to the surrounding tissue, do not activating a chronic immune response[13]. Hydrogels based on natural biopolymers have potential advantages over synthetic polymers, such as excellent biocompatibility, possible bioactive motifs encoded in their chemical structures[18] and they have similar properties of native ECM. However, the

inherent variability due to their biological origin and possible immunoresponse makes natural hydrogels more difficult to work with from a clinical viewpoint[19].

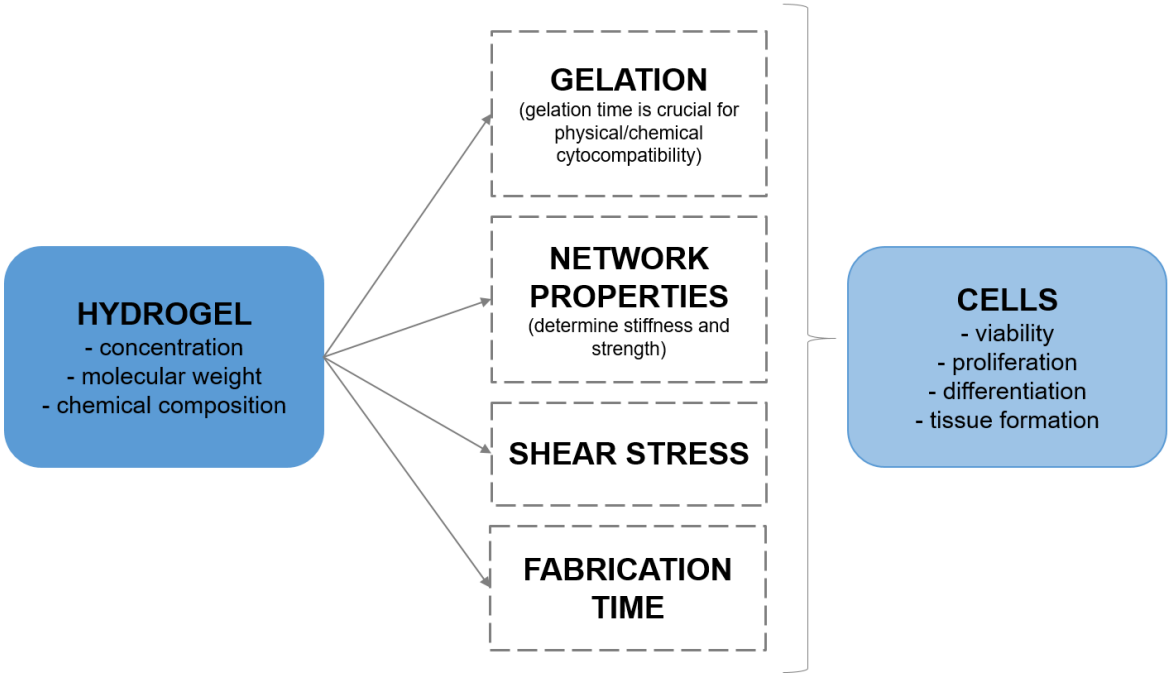

Figure I.2. Schematic representation of the parameters that influence some properties of polymeric hydrogels. The polymer concentration, molecular weight and chemical composition directly influence gelation process, fabrication time, shear stress and network properties. These properties will influence cell viability, proliferation and differentiation.

The great biological performance of some materials such as collagen or Matrigel as a cell-culture substrate and in angiogenesis assays makes them a gold standard for cell culturing. Although the great biological response in terms of cell adhesion and proliferation there are important concerns related to their animal origin and difficult manipulation. Researchers have been looking for alternatives to overcome certain limitations of Matrigel and collagen. Some works rely on the use of synthetic inert materials that may be further functionalized with specific bioactive domains while others focus on the use of human derived materials. Despite the design flexibility for specific applications, synthetic systems are typically expensive and their synthesis can be time consuming, requiring often multiple processing steps. In the current era, striving toward personalized medicine and targeted therapy, the development of materials that mostly mimics the *in vivo* environment is one of the most important topics to address[20,21]. The aim of this project is the development of biological relevant materials that could be used

as an enabling platform for personalized tissue engineering or drug discovery. This will be performed using human platelet rich plasma as raw material to produce the hydrogels.

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Chapter II*

Biomaterials from platelet-rich plasma for tissue engineering and regenerative medicine

* This chapter is based on the following publication:
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Biomaterials from platelet-rich plasma for tissue engineering and regenerative medicine

Santos SC[1], Sigurjonsson ÓE[2], Custódio CA[1*], Mano JF[1*]

1- Department of Chemistry, CICECO – Aveiro Institute of Materials, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal
2- Department of research and development. The Blood Bank, Landspitali - University Hospital. Reykjavik University, Snorrabraut 60, 105 Reykjavik, Iceland

* Corresponding author: catarinacustodio@ua.pt and jmano@ua.pt

Abstract

Platelet-rich plasma (PRP) and its derivatives have been widely investigated and applied in regenerative medicine. The use of PRP as a supplement of cell culture media has consistently shown to potentiate stem cell proliferation, migration, and differentiation. In addition, the clinical utility of PRP is supported by evidence that PRP contains high concentrations of growth factors (GFs) and proteins which contribute to the regenerative process. PRP based therapies are cost effective and also benefit from the accessibility and safety of using the patient's own GFs. In the last years, it has been witnessed a great development on PRP based biomaterials, with both structural and functional purposes. Herein, we overview the most relevant PRP applications encompassing PRP based materials for tissue engineering and regenerative medicine. This review also summarizes the challenges in the field and provides a perspective on future directions in this field.

1. Platelets

Platelets are a natural reservoir of multiple proteins and growth factors (GFs) stored in the α-granules. *In vivo*, platelets are activated at sites of tissue injury, where they promote tissue repair, including revascularization. Therefore, through lysis of human platelets, GFs can be extracted, providing a less expensive and safer alternative to the use of recombinant or animal proteins and GFs. For decades, platelet concentrates have been a standard

transfusion component for the treatment of clotting disorders[1]. The platelet concentrates are transfused to the patients who suffer from thrombocytopenia or some other platelet disorder in order to sustain the hemostatic function. The use of platelets has been increasing in the last 30 years, due to advanced surgical techniques and therapies. Nowadays, they are considered vital products in blood banking[2,3].

Platelets are not only involved in maintenance of human body but also play an important role in the coagulation and healing processes[2,3]. When activated (figure II.1) by substances such as thrombin, calcium chloride or collagen[4], they release some molecules present in the α-granules, including GFs and multiple cytokines. Such molecules, when in contact with cell transmembrane receptors promote expression of genes involved in processes like cellular recruitment, growth and morphogenesis[3]. Dense granules contain serotonin, histamine, dopamine, calcium and adenosine. Serotonin and histamine, when released, increase capillary permeability recruiting inflammatory cells to the site of injury and adenosine binds to its receptor in order to modulate inflammation during wound healing. Dopamine works as a regulator of heart rate and blood pressure and calcium is a cofactor for platelet aggregation[2].

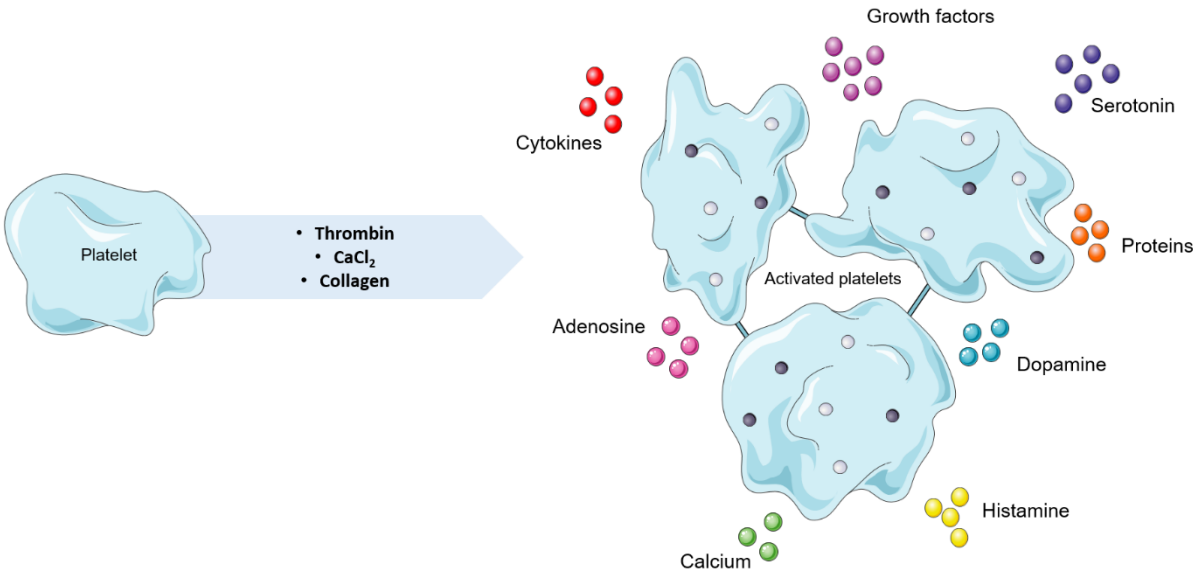

Figure II.1. Platelet activation process. Substances like thrombin, calcium chloride and collagen can activate platelets. Activated platelets release some molecules like cytokines or growth factors that are involved on cellular recruitment, growth and morphogenesis.

Platelet-rich plasma (PRP) is an autologous source of platelets. In PRP the normal concentration of platelets is at least $1x10^6$ platelets/μL in 5 mL of plasma, while in whole

blood range from $1,5x10^5$ platelets/μL to $3,5x10^5$ platelets/μL[2,5]. PRP, as a "cocktail" of multiple GFs, may be used *in vitro* to better mimic of what happens *in vivo*. First reports on the use of PRP as a possible therapy are from 1990s, with its application in cardiac surgery[6] and also in bone grafts[7]. Nowadays, there are several areas where the potential therapeutic effect of PRP is documented: dentistry[7–13], orthopedics[3,14,15], neurology[16,17], plastic surgery[18], musculoskeletal conditions[19] and also *in vitro* fertilization[20].

2. Preparation of platelet-rich plasma and platelet lysates

PRP is obtained from whole blood samples by a differential centrifugation process (Figure II.2). Only two spins are used: after the first spin (high speed) two layers are obtained – one is platelet poor plasma (PPP) and the second one is the mixture of red blood cells and PRP; after a second spin of 10 minutes (low speed) three layers are obtained – one is PPP, the second one is PRP and the third are red blood cells[4,21,22].

Human platelet lysates (PL) are obtained from platelets present in PRP. PRP is treated with freeze/thaw cycles for platelets disruption and release of their content (e.g. proteins, GFs and cytokines)[23] (Figure II.2). Standardized freeze/thaw cycles of -80ºC and 37ºC have been widely used for the PL preparation. Nevertheless, recent study suggests −196°C/4°C cycles for increased concentration of GFs[24]. Additionally, there are other processes described in the literature for the preparation of PL such as calcium chloride and thrombin activation, still freeze/thaw technique appears to be more effective[5].

3. Biochemical composition of platelet-rich plasma

GFs present in human PRP include platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF)[25], insulin-like growth factor I and II (IGFI and II), transforming growth factor β (TGF-β)[26], epidermal growth factor (EGF), epithelial cell growth factor (ECGF)[27], connective tissue growth factor (CTGF), platelet factor 4 (PF4), interleukin 8 (IL-8) and keratinocytes growth factor (KGF)[28]. These GFs perform functions such as chemoattraction, cell proliferation and maturation, matrix synthesis and angiogenesis[2,4].

Some of these factors are also present in PL such as PDGF, TGF- β, IGF, FGF, EGF, VEGF and ECGF[2]. Other components present in PL are fibrinogen that plays an important role in blood clotting cascade and fibronectin that affects cell adhesion, growth, migration

and differentiation[2,4]. Also clotting factors are present – factor V and Va, factor XI, protein S and antithrombin – and all of them are responsible for thrombin activation and fibrin clot formation[2].

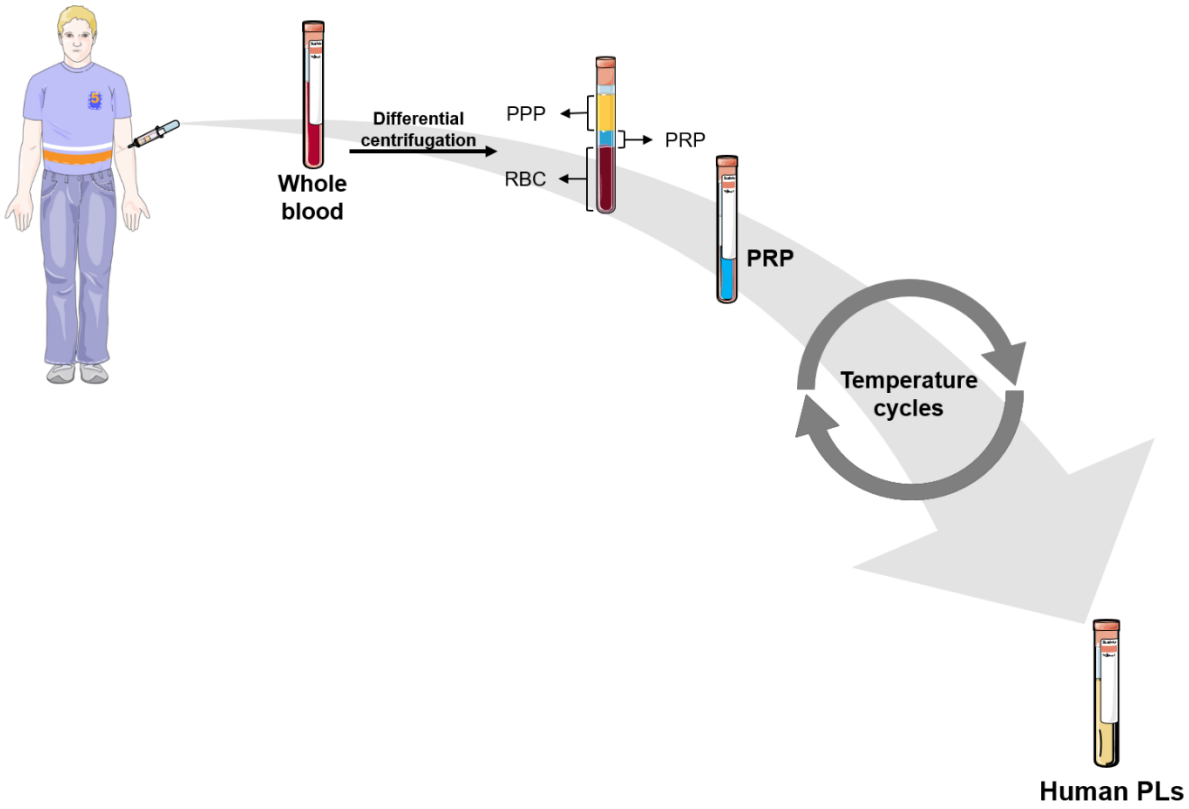

Figure II.2. Schematic representation of PRP isolation and PL preparation by freezing and defrosting technique.

4. Human platelet-rich plasma as an alternative for animal-derived serum

Traditional protocols for the isolation and *in vitro* culture of cells commonly involve culture media supplemented with fetal bovine serum (FBS). The search for alternatives to the use of FBS in *in vitro* cell culture has become a major goal in terms of Good Manufacturing Practice (GMP), to ensure animal product-free conditions. There are also the ethical issues associated with the collection methods of animal serums and potential limits of availability[29,30]. For clinical-scale manufacturing, serum-free and xenogeneic-free formulations have also been suggested as alternatives to FBS-supplemented media. PL has been suggested as an efficient alternative to FBS for cell and tissue expansion, reducing the risks of transmission of xenogeneic contaminants as virus, bacteria and prions, as well as

xenogeneic antigens. Increased safety for cell therapy protocols and cost reduction are the major benefits of human alternatives when compared to animal-derived mediums or mixtures of recombinant GFs[31]. The influence of different supplements for *in vitro* cell culture was studied by V. Mirabet *et al* where they conclude that cell-based therapies can benefit if products from human origin like PL are used as they can avoid the risk of xenogeneic infections[32].

	Molecule Category	**Molecule**	**Biologic Activity**
PRP	Growth Factors	TGF-β1, bFGF, PDGF-A and B, PD-EGF, VEGF, ECGF, IGF-I and II CTGF and KGF	Matrix synthesis, chemoattraction, cell proliferation and maturation, increase angiogenesis and vessel permeability, mitogenesis regulation, collagen synthesis and collagenase secretion regulation, fibrosis, platelet adhesion, pro-inflammatory mediation
	Cytokines and chemokines	PF4 and IL-8	Chemoattraction, cell recruitment, pro-inflammatory mediators
Human platelet α-granules	Growth Factors	TGF-β1 and 2, PDGF, IGF-I and II, bFGF and FGF-2, EGF, VEGF-A and C, HGF and ECGF	Matrix synthesis, chemoattraction, cell proliferation, differentiation and maturation, angiogenesis
	Cytokines and chemokines	RANTES, IL-8, MIP-1α, GRO-α, ENA-78, MCP-1, 2 and 3, IL-1β, CXCL6, SDF-1α, PF4, neutrophile chemo active protein	Chemotaxis, cell proliferation and differentiation
	Adhesive proteins	Fibrinogen	Blood clotting cascade
		Fibronectin	Affects cell adhesion, growth, migration and differentiation
	Clotting factors	Factor V/Va, XI, protein S and antithrombin	Thrombin activation and fibrin clot formation

Table II.1. GFs, cytokines and chemokines present in PRP and GFs, cytokines/chemokines, adhesive proteins and clotting factors present in human platelet α-granules[1,2,28,39,40].

Studies with different cell types have shown that cells cultured *in vitro* with PL as medium supplement proliferate better when compared with cultures using FBS as a supplement. Cells from bone marrow[32], adipose tissue[32,33], trabecular bone[32], dental pulp[32], mesenchymal stem cells[34,35], human articular chondrocytes[33], myofibroblasts[36], human immortal keratinocyte cell line[37] and osteoblasts[38] respond to the use of PL with increased proliferation rate. It was also reported that the use of PL instead of FBS may have an influence in cell morphology. For example, when osteoblasts, cartilage cells, bone marrow cells and mesenchymal stem cells are cultured with PL, cell morphology is affected possibly due to the higher proliferation rate[32,35,38]. Apparently, this do not interfere with the differentiation potential and immunophenotypic characteristics, at least in case of mesenchymal stem cells[31].

The proliferation process is influenced by the presence of cytokines, GFs and attachment factors. These molecules are responsible for cellular migration, redistribution, adhesion and proliferation and they are present in PL. This is a reason why cells show higher proliferation rates when PL are used as a supplement in culture media[21]. PL have a total protein content typically exceeding 50-55 mg/mL while for example FBS composition include low and high molecular weight proteins and protein content is on average 38 mg/mL. Despite the promising results, a major concern regarding the use of PRP is their heterogeneity and demanding characterization. Advanced characterization techniques such as mass spectrometry and two-dimensional gel electrophoresis analyses could be important for a better characterization and standardization in the use of PL[29].

5. Platelet-rich plasma and fibrin based materials

Liquid PRP contains fibrinogen, which is transformed in a fibrin matrix by thrombin cleavage (figure II.3). *In vivo*, fibrin acts as a scaffold for tissue repair and provides important cues for directing cell phenotype following injury. This matrix can be broken down by plasmin enzyme through a process called fibrinolysis[41]. Based on this phenomena, researches have been exploring fibrin gel preparation *in vitro* by the addition of thrombin and calcium to PRP/PL. One of the first commercial applications of fibrin gels was in the development of fibrin sealants. This type of sealants are available in Europe since 1972, and in the last years a number of novel fibrin sealants have been approved by the Food and Drug Administration for use as haemostats in surgery and adhesives for skin graft attachment[42].

Fibrin matrix forms aligned fibrils in direction of extension and perpendicular to the direction of compression, with mechanical properties suitable for vascular tissue engineering[43]. J. N. Long and R. T. Tranquillo developed materials with organized elastic fibers formed when neonatal rat smooth muscle cells were cultured in fibrin gels[44]. Their findings suggest that fibrin-based constructs can be used as a model system for the study of three-dimensional (3D) elastic structures. The combination of fibrin gels with hyaluronic acid was proposed by T. N. Snyder *et al* as a suitable 3D environment for the proliferation of bone marrow-derived mesenchymal stem cells[45]. Recently, Marcinczyk *et al* proposed fibrin based gels supplemented with laminin as tissue engineered constructs for skeletal muscle regeneration[46].

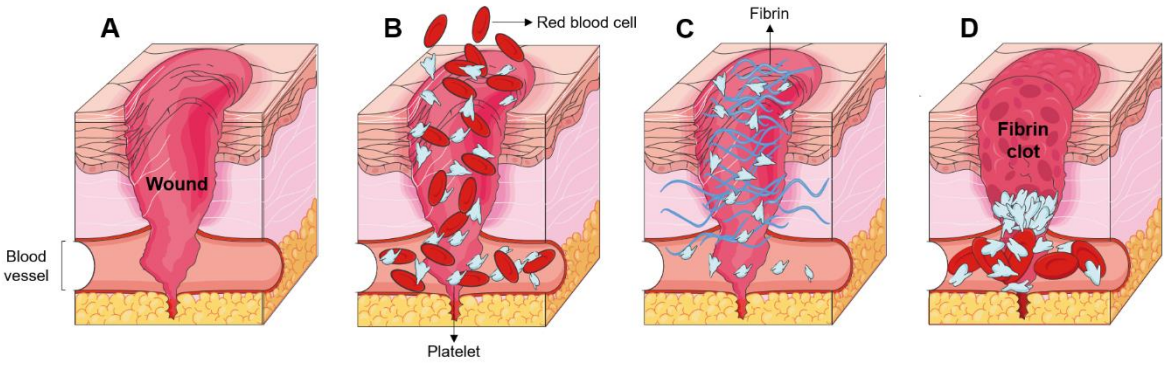

Figure II.3. Fibrin clot formation process. In response to a tissue wound (A) platelets start to aggregate at the injury site (B). Coagulation cascade is activated and fibrinogen is transformed in fibrin by thrombin cleavage (C). The end of the process is a fibrin clot formation (D).

As previously referred in this review, PL based gels are usually formed by the addition of calcium and thrombin that triggers a coagulation cascade, promoting fibrinogen polymerization into fibrin clots. G. Walenda *et al* reported a PL based gel that provides a 3D scaffold for mesenchymal stromal cells expansion by addition of calcium ions to PL[26]. T. M. Fortunato *et al*[47] and S. T. Robinson *et al*[48] described the fabrication of PL gels and reported that PL based gels have the capacity to efficiently release GFs essential at promoting cell adhesion and proliferation. They also demonstrate that these gels support the formation of fully human capillary network, establishing a *de novo* endothelial network and lumen present in cappilaries[47], and led to a rapid neovascularization of ischemic tissues[48]. The results from these works reveal that PL gels have unique structural properties that allow it to behave as a viscoelastic solid with improved mechanical properties compared to fibrin only hydrogels. Furthermore, mechanical measurements have shown that PL gels can have a very soft and

viscous consistency with increasing elasticity with the increase of PL percentage[26]. Despite all the great intrinsic properties of PL based gels, this type of materials have poor mechanical properties and poor stability *in vivo* and cell culture conditions[49]. The development of new crosslinking methods is therefore a need. Considering this, P. Babo *et al* reported the fabrication of PL-based membranes by crosslinking PL proteins with genipin[27]. These membranes show particularly high stiffness and elasticity, important characteristics for regeneration of soft tissues, and a long-term stability, which is an uncommon characteristic of PL based biomaterials. The authors also demonstrated the potential of these membranes to act as a substrate for stem cell attachment and growth and to provide a propitious environment to cells development because of its richness in GFs. From these membranes, P. Babo *et al* made multipatterned devices with six different patterns and studied periodontal ligament cells proliferation, migration and alignment[50]. This technique was shown to be an effective strategy for improvement of periodontal regeneration tissue, as they promote the proliferation of periodontal ligament cells.

6. Hybrid platelet-rich plasma based materials

PRP and PL have been combined with different biopolymers not only to improve the mechanical properties of PRP/PL based materials but also to render the biopolymers bioactive and mimic native extracellular matrix or for the controlled delivery of GFs. This may represent important biological and economic advantages[51,52], as recombinant GFs widely used are very expensive and may promote immunogenic reactions[52,53]. Methods to create 3D matrices with incorporated PL include incubation of the polymeric matrices with PL solutions or mixing PL with the polymeric precursor before curing[21,54].

For many applications there is a need for chemically crosslinked or hardened biomaterials. Since most of the natural based polymers do not crosslink in a stable structure at body temperature, it is necessary to stabilize these materials by establishing chemical crosslinks between the polymer chains. Chemical crosslinking typically relies in reagents such as genipin, glutaraldehyde or carbodiimides. The incorporation of moieties that allow for photoinduced crosslinking, is also attractive and have been widely explored in the synthesis of materials for biomedical applications.[55–57]. This type of modification results in materials that are easily crosslinked by light irradiation avoiding the use of toxic components, with tunable mechanical properties and stable at cell culture conditions and *in*

vivo. For example, X. Liu *et al* developed an *in situ* photocrosslinkable PRP – complexed hydrogel glue by combination of a photoresponsive hyaluronic acid group with PRP[58]. Results showed that it is a robust hydrogel scaffold that provides a controlled release of GFs and strong tissue adhesive ability. *In vivo* tests demonstrated that this PRP derived hydrogel can promote hyaline cartilage regeneration. Controlled release of GFs is an important characteristic of PRP/PL biomaterials as this property increases therapeutic efficacy of these materials.

R. Ito *et al* proposed a collagen/gelatin scaffold impregnated with PL which accelerates formation of dermis-like tissue in mice[59]. Their findings also indicate that collagen/gelatin scaffold bind to TGF-β and PDGF-BB and release these GFs in a sustained manner. V. E. Santo *et al*, have shown that, the incorporation of PL in gellan gum gels significantly improved cellular adhesion and viability in the 3D constructs[25]. The use of microfluidic tools allowed the design of a fiber-like hydrogel incorporating a gradient of PL along the length of the fiber. A hydrogel based on PL loaded chitosan-chondroitin sulfate nanoparticles was also developed by the same authors[60]. Biological assays showed that the hydrogels maintained cell viability and that PL can work as a GFs supplement for cell proliferation. The use of PL to build these aggregates can also provide mechanical stability to the 3D constructs. Such type of results strengthen the potential of using PRP/PL in order to provide bioactive signals could enhance a therapeutic effect but could be also a structural element in the development of biomaterials.

Several works reported on the influence of PL in proliferation, growth and differentiation of many cell types, including mesenchymal stem cells, adipose derived stem cells or endothelial cells. For example, J. Leotot *et al* studied how PL can influence mesenchymal stromal cells behavior if used as a coating of hydroxyapatite/β-tricalcium phosphate scaffolds[61]. Not only a higher number of mesenchymal stromal cells adhered to the scaffold but also cells had increased cytoplasmic extensions after 3 hours in culture. After 24 hours in culture, cells showed an elongated shape covering almost entirely the scaffold and 7 days later mesenchymal stromal cells completely covered the biomaterial. Proliferation and differentiation of adipose derived stem cells (hASCs) are also influenced by the presence of PL. S. Oliveira *et al* have shown that PL and marine-origin polysaccharides assembled by layer-by-layer and shaped into fibrils by freeze-drying over 3D scaffolds induced hASCs differentiation into mature osteoblasts[52]. They also observed

that the fibrillar structures with PL allowed a 3D organization of the mineralization. This type of hierarchal scaffolds combined with PL demonstrated also pro-angiogenic properties, promoting formation of tubular structures in endothelial cells[62]. L. S. M. Teixeira *et al* studied the incorporation of PL in dextran-tyramine hydrogels to provide chemo-attractant properties and trigger chondrogenic differentiation of mesenchymal stromal cells[63]. Their results suggest that dextran hydrogels complemented with PL can potentially be used as a cell-free approach to repair cartilage defects. Using a more selective strategy C. A. Custódio *et al* reported the synthesis of chitosan microparticles functionalized with PL derived GFs[64]. The functional particles assembled into a stable 3D construct triggered by the presence of hASCs, which act as binder agents and induce the formation of a hydrogel network.

Overall, the incorporation or blending of PRP/PL in biomaterials may be a key way to increase the therapeutic efficacy of such GFs sources, as well to improve the mechanical stability of PRP/PL based materials.

7. Clinical applications of platelet-rich plasma

The clinical application of PRP has been documented in many fields. In particular, recent works described that PRP improves the wound healing process, being able to promote tissue regeneration and accelerate bone formation. An example of this, is the work from A. Patel *et al* that showed the positive influence of PRP in the wound healing process in patients submitted to cardiac surgery. The use of PRP decreased the incidence of infections derived from sternotomy[65]. Such results suggest that the use of PRP may decrease the incidence and costs of sternal wound complications following cardiac surgery.

In recent years, PRP injections have been used as treatment of musculoskeletal conditions like rotator cuff tears[66], knee osteoarthritis[67], ulnar collateral ligament tears[68], lateral epicondylitis[69], hamstring injuries[70] and Achilles tendinopathy[71]. Total knee arthroplasty is a common orthopedic procedure for restoring knee function. M. J. Gardner *et al* studied the efficacy of PRP in pain control and blood loss in this procedure[15]. They concluded that the group who receive PRP treatment required less drugs, achieved higher functional range of motion earlier than control group and also had a lower postoperative decrease in hemoglobin. Also W. J. Berghoff *et al*[14] showed that PRP, when used in total knee arthroplasty, helps in the recovery process. A. K. Mishra *et al* evaluated the use of PRP treatment in patients with chronic tennis elbow[72]. Results showed that after 24 weeks PRP-

treated patients had an improvement in their pain scores as also in elbow tenderness. Surgery recovery process are difficult and painful, however, such results showed that these can be overcome if PRP is used as a treatment.

A number of studies have focused on how PRP and PL affect osteoblasts, osteoclasts and mesenchymal osteoprogenitor stem cells so it can be considered a therapeutic product for bone and cartilage repair. N. Labibzadeh *et al* were pioneers reporting the treating of long bone nonunion[73]. By implanting mesenchymal stromal cells in combination with PL into bone injury sites of seven patients, the authors concluded that harvest, isolation and implantation of mesenchymal stromal cells combined with PL is safe for treating bone nonunion. Despite its use, there has been conflicting evidence about the effectiveness of PRP in treating bone and cartilage[74,75]. More studies are needed to determine the efficacy of using PRP to manage musculoskeletal disorders.

PRP is widely known because of its capacity of improving wound healing process, S. Suryanarayan *et al* carried out a study to evaluate the efficacy of using autologous PRP in chronic nonhealing leg ulcers treatment[76]. Patients with leg ulcers submitted to a treatment with PRP showed that not only PRP helps in healing process but also it helps in pain reduction. K. Kazakos *et al* studied the efficacy of a PRP gel in acute limb soft tissue wounds, demonstrating that in patients treated with PRP gel healing process was faster[77].

Progressive hemifacial atrophy is a degenerative condition characterized by a slow and progressive atrophy of one side of the face. The pathogenesis of the syndrome is not clearly understood, however V. Cervelli and P. Gentile[78] studied the influence of PRP in this pathology. The treatment with PRP mixed with fat tissue favors tissue growth and also 3D face reconstruction.

Most recently, the application of PRP in neuropathies has been studied. Although most of the studies are *in vitro* and *in vivo*, there are already some clinical trials showing that PRP injection could be an improvement on neuropathies recovery[79]. S. Anjayani *et al* carried out a study on the effectiveness of PRP injections in patients with leprosy peripheral neuropathy[80]. PRP injection was able to stimulate nerve regeneration and also promote pain reduction, which means that PRP injections in perineural nerves of leprosy peripheral neuropathy patients it's a good way to improve their recovery.

Overall, these results indicated that PRP has a great potential in different clinical applications. Nevertheless, more scientific research with well design studies and larger samples sized will have to conclude PRP effectiveness before it is more widely covered.

8. Platelet-rich plasma market assessment

PRP market was evaluated at US$ 201.2 million in 2016 but it is expected to grow in the next years[81]. Nowadays autologous PRP segment dominates the global PRP market, however, it is expected that allogenic PRP segment grows up because of its cost effectiveness and safety. PRP application is still dominated by orthopedic segment but its application in other areas is emerging very quickly. Clinical areas such as neurology, plastic surgery and general surgery may become major users of this therapy.

Assessing the different types of PRP we can obtain, namely pure PRP, leukocyte-rich PRP, pure platelet-rich fibrin and leukocyte-rich platelet-rich fibrin, pure PRP is the most preferred PRP type, accounting for over 40% share of the overall market. The largest market for PRP in 2016 was North America with a share of 43.3% followed by Europe, the second largest share of the global PRP market in 2016. The top five players of this market are Zimmer Biomet, Stryker Corporation, DePuy Synthes, Harvest Technologies and Arthrex, Inc, accounting for more than 70% of the total market share in 2016. Currently, most of these key players are domiciled in North America, nevertheless they are focusing on expanding and be presence in a larger geographic area, mainly in Asia Pacific which is the most lucrative market.

9. Conclusions and future directions

The patient's own plasma, PRP and derivatives as therapeutics have found application in many fields. An advantage of PRP therapies is the simulation of the physiological repair process by releasing autologous GFs. PRP is a promising alternative to animal derived supplements based on the efficacy of autologous GFs to accelerate tissue healing, allowing a fast recovery after muscles, ligaments, tendon or cartilage lesions. Still, depending on the preparation technique, PRP composition may be affected. This means that more research is need for the development of standardized protocols. An improved characterization of this complex mixture of biomolecules is also imperative and the application of advanced proteomic techniques for an effective categorization of the protein and GFs content could be

particularly useful on that. PRP is indeed an excellent source of GFs and signaling molecules and several works have been reporting on their use in wound healing and tissue regeneration. The use of autologous PRP and cells is now an effective alternative in regenerative medicine and tissue engineering. In the case of biomaterials based on PRP or combined with PRP and its derivatives, they possess attractive bioactivity but weak mechanical properties. New strategies to improve the *in vitro* and *in vivo* stability of these materials are being explored. Finally, from a structural point of view, new strategies will be required to produce PRP-based devices with improved mechanical properties for load bear applications and with tuned degradation rates that could follow the regenerative process.

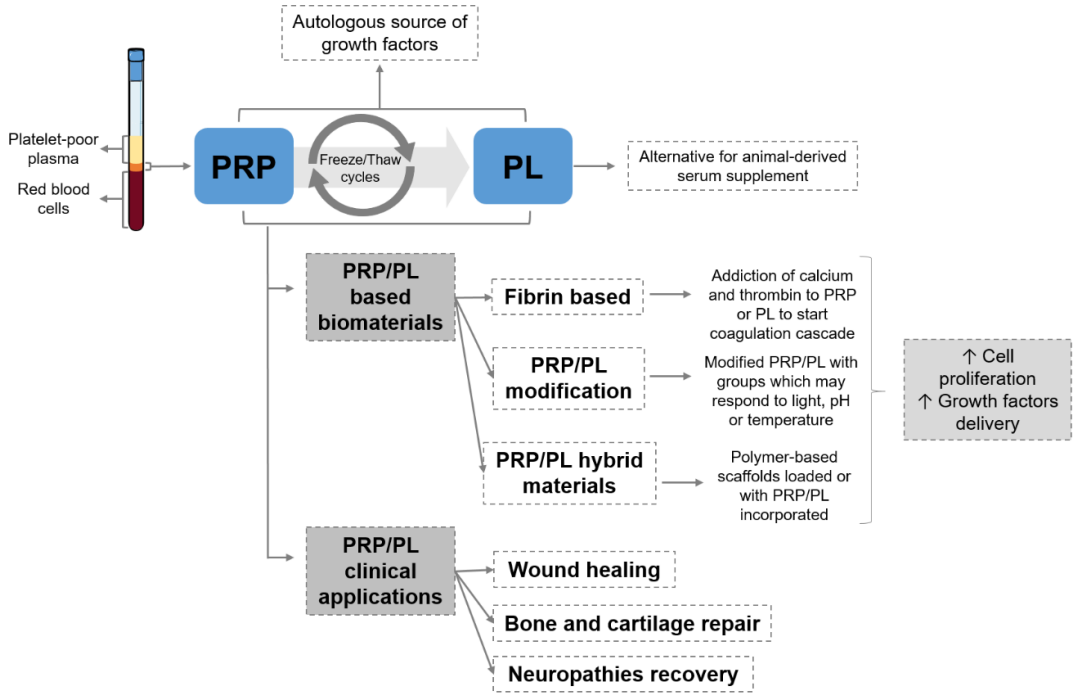

Figure II.4. PRP/PL acquisition process and PRP/PL based biomaterials and clinical applications.

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Chapter III

Materials and methods

1. Platelet lysates

Platelets are an important source of growth factors (GFs), cytokines and other molecules involved in tissue regeneration[1]. Platelet-rich plasma (PRP) is obtained from a whole blood sample by differential centrifugation and has high platelet concentration, at least $1x10^6$ platelets/μL in 5 mL of plasma[2,3]. Platelet lysates (PL) are obtained from PRP by freeze/thaw cycles in order to allow platelet disruption[4,5]. PRP and PL are both autologous sources of GFs and other molecules involved in cell proliferation and differentiation and have been used as a possible therapy since 1990s[6,7]. In this work PL (STEMCELL Technologies, Canada) were stored at -20ºC and thawed in a 37ºC water bath with low agitation before use.

2. Chemical modification of platelet lysates

Since many polymers are not reactive in its native structure, insertion of chemical modifications in order to make them responsive to a stimulus like pH changes, light or temperature is a widely used procedure for development of new materials[8–13].

Methacrylation of polymeric backbones is one of the possible polymer modifications. It can be performed with reagents such as glycidyl methacrylate, methacryloyl chloride or methacrylic anhydride (MA). MA, which was used in this work for PL modification, seems to provide greater control over the crosslinking density, because methacrylate groups restrict the maximum crosslinking density achievable independent of the amount of initiator added or experimental conditions like time and temperature[8–10,14].

Methacrylamide and methacrylate groups can be inserted in a protein/peptide chain, as figure III.1 shows. The methacryloyl groups added to the polymeric backbone are functional groups capable of covalent crosslinking by free radical polymerization in presence of a photoinitiator upon ultraviolet (UV) radiation [9,15,16].

In order to make PL photoresponsive, in this work, a chemical modification using MA 94% (Sigma-Aldrich, Germany) was performed. The PL reaction with MA was performed at room temperature during 4 hours and with pH maintained between 6-8, in order to achieve methacryloyl PL (PLMA). After 4 hours of reaction, the PLMA were purified by dialysis with Float-A-Lyzer G2 Dialysis Device 3.5-5 kDa (Spectrum, USA) against deionized water during 20-24h to remove the excess of MA. The PLMA solution was filtered with a 0.2 μm filter in order to sterilize it, frozen with liquid nitrogen, lyophilized and stored

at 4ºC until further use. Two degrees of modification were achieved: low-degree of modification (PLMA100) and high-degree of modification (PLMA300).

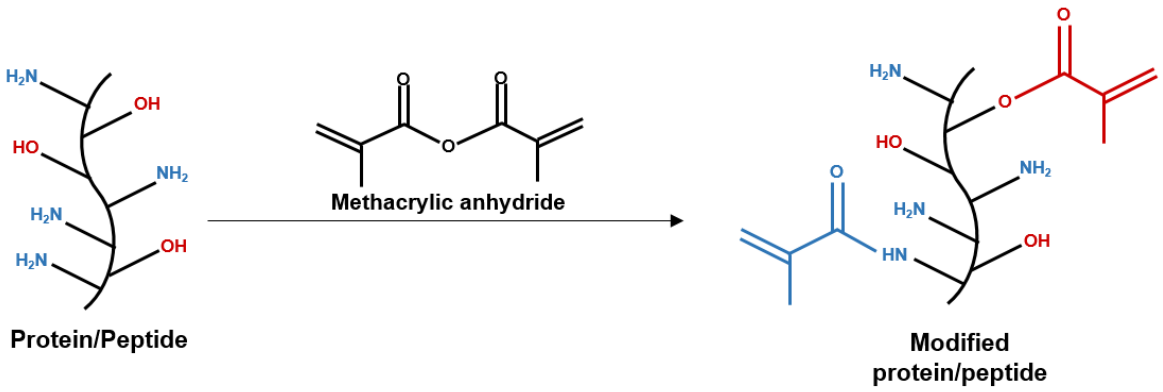

Figure III.1. Protein/peptide modification with methacrylic anhydride. Methacrylamide and methacrylate groups inserted in protein/peptide chain will allow photopolymerization.

3. PLMA characterization

3.1. ^{1}H NMR

NMR analysis were made in order to verify the functionalization of original PL. Methacryloyl groups can be detected by ^{1}H NMR spectroscopy since these groups have double bound protons and also protons from the methyl group that can be detected. ^{1}H NMR analysis of methacrylated polymers prove the methacrylation by the appearance of distinctive peaks in the double bond region at δ 5.2 – 5.6 ppm and a peak that corresponds to the -CH_3 of the methacrylate groups at δ ≈ 2 ppm[16]. For PL and PLMA characterization by ^{1}H NMR, solutions of 10 mg/mL in dimethyl sulfoxide (DMSO) (Fisher Scientific, USA) were prepared and analyzed with 18 seconds relaxation delay and 300 scans. The ^{1}H NMR spectra were recorded on a Bruker AMX 300 spectrometer at 300.13 MHz.

3.2. Mass spectrometry

Mass spectrometry has become a powerful method for protein characterization. Protein can be modified in order to module their function and characterization of modified proteins can be done by mass spectrometry. This characterization is possible due to the increase or decrease of the molecular mass of the affected amino acid residue detected by mass spectrometry analysis[17]. In this work, identification of proteins and modified proteins

in PL and PLMA, respectively, was performed on a TripleTOF 6600 mass spectrometer after protein digestion with trypsin.

4. Hydrogel formation

Photocrosslinking is a simple technique used to fabricate three-dimensional (3D) hydrogel networks[9] by using a light source and a photoinitiator or crosslinker molecule[18,19]. This technique require light exposure of a solution with crosslinkable components to induce covalent crosslinking between neighboring chains[18]. For some time now, photosensitive biomaterials have been developed providing a platform in which application of light can be used to introduce control of crosslink formation[12]. It is a commonly applied method and until now there are no reported adverse effects on cell viability and function[10,20]. Photocrosslinking has some advantages compared to other crosslinking techniques because it is cost-effective, rapid, can be carried out under mild conditions and allows fabrication of very controlled shape and size hydrogels [9]. It also may limit the need for invasive surgical procedures because it allows to a trans-tissue *in situ* polymerization after the material is injected or placed in the injury site and irradiated[19,21].

Addition of groups like acrylate moieties to the polymeric backbone can be used to provide light polymerization in order to obtain a hydrogel. Therefore, in this work, PLMA when in solution and by UV irradiation are able to form hydrogels. Lyophilized PLMA were dissolved in a solution of 0.5% (w/v) 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone (Sigma-Aldrich) in PBS (Sigma-Aldrich) to final concentrations of 10% or 15% (w/v) PLMA. Hydrogels were prepared by pipetting the polymer solution to polydimethylsiloxane (PDMS) (Dow Corning, USA) molds with 3.5mm or 6mm diameter followed by UV irradiation (1.54 W/cm^2) during 60s.

5. PLMA hydrogels characterization

5.1. Mechanical properties

Mechanical properties of hydrogels play a key role in cell behavior and are important characteristics in tissue engineering approaches. The mechanical properties are dependent of the used polymer, crosslinking density and swelling[22,23]. These parameters may be adjusted in order to prepare the appropriate hydrogel functions and promotion of new tissue formation. Cell adhesion and proliferation processes are heavily influenced by hydrogel mechanical properties[22]. Some changes in hydrogel stiffness may affect oxygen and nutrient

permeability, availability of binding sites and water content[24]. The mechanical behaviour of PLMA hydrogels was characterized on the basis of compression tests employing a Universal Mechanical Testing Machine Shimadzu MMT-101N (Shimadzu Scientific Instruments, Kyoto, Japan) equipped with a load cell of 100N. To this end, both unidirectional and cyclic compression assays were performed at room temperature on as-prepared cylindrical hydrogels specimens with a diameter of 3.5 mm and height of 2 mm. The nominal stress was obtained by dividing the compressive load by the initial (uncompressed) cross-sectional area of the specimen. The Young's modulus was defined as the slope of the linear region of the strain/stress curve, corresponding to 0–25% strain. Ultimate stress and ultimate strain values were taken as the point where failure of the hydrogel occurred.

5.2. Scanning electron microscopy

Scanning electron microscopy (SEM) analysis was performed in order to analyze the hydrogels structure. SEM images were also used to study the porosity of the hydrogels by measuring the pore size using Image J software. The hydrogels were frozen with liquid nitrogen and then lyophilized. Samples were sputter-coated with gold and imaged via SEM using Ultra-high Resolution Analytical Scanning Electron Microscope HR-FESEM Hitachi SU-70 (Hitachi, Tokyo, Japan).

5.3. Water content

Water content is an important property of hydrogels. In this work, hydrogel samples of PLMA100 and PLMA300 at 10% and 15% (w/v) were made in triplicate and immersed in 5 mL of PBS. Samples were incubated at room temperature during 2 hours. After 2 hours the wet weight (w_w) was measured and samples were frozen and lyophilized. After lyophilization, dry weight (w_d) was measured and compared with the initial wet weight. The water content was calculated according to following equation:

$$\text{Water content (\%)} = \frac{w_w - w_d}{w_w} \times 100$$

6. Quantification of proteins and growth factors release

PRP and PL have high content of GFs[3,25,26] and therefore PLMA hydrogels can be a source of GFs derived from used PL. For GFs release assays, six samples of each PLMA100

at 10% and 15% (w/v) were made. Samples were added to falcons with 5 mL of PBS (Thermo Fischer Scientific, USA). The samples were incubated in a bath at 37ºC with constant agitation (60 rpm). At each time point, an aliquot of 600 µL was taken from each sample and 600 µL of fresh PBS added. Collected samples were stored at -20ºC. Total protein quantification was performed with Micro BCA Protein Assay Kit (Thermo Fisher Scientific). Quantification of specific proteins and GFs was performed by ELISA assay.

7. *In vitro* cell encapsulation

7.1. Cell culture

The biological performance of PLMA hydrogels was assessed using L929 Cell Line (European Collection of Authenticated Cell Cultures (ECACC)) and human adipose stem cells (hASCs) (ATCC).

L929 cells were cultured in DMEM low glucose, supplemented with sodium bicarbonate, 10% FBS and 1% antibiotic/antimycotic. Cells were used between passages 3 and 25.

Stem cells are capable of self-renew and differentiation along multiple lineage pathways what makes them really interesting for tissue engineering area. hASCs and other stem cells are promise as a therapy and treatment of a wide range of medical conditions. hASCs can be isolated from adipose tissue, which is an abundant source of hASCs capable of differentiate in multiple lineages[27]. hASCs were cultured in Minimum Essential Alpha Medium supplemented with sodium bicarbonate, 10% of FBS and 1% antibiotic/antimycotic. hASCs were used until passage 5. Cell suspensions were prepared by trypsinization (trypsin/EDTA solution, Sigma-Aldrich). The cells were incorporated into the PLMA solutions to a final concentration of $2x10^6$ cells/mL in the case of L929 and $3x10^6$ cells/mL for hASCs. The hydrogels were prepared as described previously in section 4.

7.2. Biological evaluation of developed materials

7.2.1. Cell viability and proliferation

7.2.1.1. Live/Dead test

Live/Dead assay was performed in order to assess the L929 and hASCs viability inside the hydrogels. This test allows the visualization, simultaneously, of the live and dead cells by fluorescence microscopy. Acetomethoxy derivative of calcein (Calcein AM) is non-fluorescent dye that easily permeates live cells where it is converted in calcein (green fluorescent die). Propidium iodide (PI) permeates damaged cells and binds to the nucleic

acids, staining them in red. At pre-determined time points, hydrogels were incubated in a solution of 2µL of Calcein AM 4mM solution in DMSO (Life Technologies, Thermo Fisher Scientific, USA) and 1µL of PI (Thermo Fisher Scientific, USA) in 1000µL of PBS at 37ºC during 30 minutes. After washing with PBS, hydrogels were examined using a fluorescence microscope (Fluorescence Microscope Zeiss, Axio Imager 2, Zeiss, Germany).

7.2.1.2. Cell metabolic activity quantification

CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, USA) was performed to evaluate cell viability inside the hydrogels. A solution of 1:10 MTS reagent:PBS was made and after wash hydrogels with PBS they were incubated with MTS solution overnight at 37ºC. The quantification was achieved by measuring absorbance at 490nm. Triplicates were made for each sample and per culturing time.

7.2.1.3. Cell proliferation by DNA quantification

DNA quantification to evaluate cell proliferation was performed using a Quant-iT PicoGreen dsDNA kit (Invitrogen, Thermo Fisher Scientific, USA). While bounded to dsDNA, PicoGreen dye is excited by UV light allowing the measurement of the produced fluorescence. Briefly, at pre-determined time-points hydrogels were washed with PBS, incubated in sterile deionized water and frozen at -80ºC. In order to induce disruption of the cells inside the hydrogel, the samples were thawed at 37ºC and placed in an ultrasounds bath during approximately 30 minutes. Standards were prepared with concentrations ranging between 0 and 2 ug/mL. The plate was incubated for 10 min in the dark and fluorescence was measured using an excitation wavelength of 480 nm and an emission wavelength of 528 nm. Triplicates were made for each sample and per culturing time.

7.2.2. Cell morphology

7.2.2.1. Immunostaining

A DAPI/Phalloidin staining was made in order to access cell morphology in hydrogels. At pre-determined time-points hydrogels were washed with PBS and fixed with a 4% formaldehyde (Sigma-Aldrich) solution during at least 2 hours. For DAPI/Phalloidin staining, a phalloidin solution (Flash Phalloidin™ Red 594, 300U, Biolegend, USA) was diluted 1:40 PBS and hydrogels were incubated at room temperature in phalloidin solution during 45 min. After PBS washes, a DAPI (DAPI (4',6-diamidino-2-phenylindole,

dihydrochloride), Thermo Fisher Scientific) solution was diluted in 1:1000 PBS and hydrogels were incubated during 5 minutes with this solution also at room temperature. DAPI binds to DNA staining in blue the nucleus and phalloidin binds to the cytoskeleton filaments showing them in red at fluorescence microscope. After washes with PBS, hydrogels were examined using a fluorescence microscope (Fluorescence Microscope Zeiss, Axio Imager 2, Zeiss, Germany).

7.3. Fabrication of PLMA microstructures

Lyophilized PLMA were dissolved in 0.5% (w/v) 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone (Sigma-Aldrich) in PBS (Sigma-Aldrich) to a final concentration of 10% (w/v). L929 cells were incorporated into the PLMA solution to a final density of $2x10^6$ cells/mL. 50µL of this solution were poured onto a glass coverslip. Then, PDMS mold was brought into contact with the solution and pressed. The mold was exposed to UV irradiation (1.54 kW/cm^2) during 60s. Finally the PDMS mold was removed from the glass and the hydrogels were cultured during 7 days. A DAPI/Phalloidin staining was performed as described in 7.2.2.1 at 7 days of incubation and PLMA hydrogels were visualized using a fluorescence microscope (Fluorescence Microscope Zeiss, Axio Imager 2, Zeiss).

8. Statistical analysis

All data were subjected to statistical analysis and were reported as a mean ± standard deviation. Statistical differences between the analyzed groups were determine by unpaired *t* test.

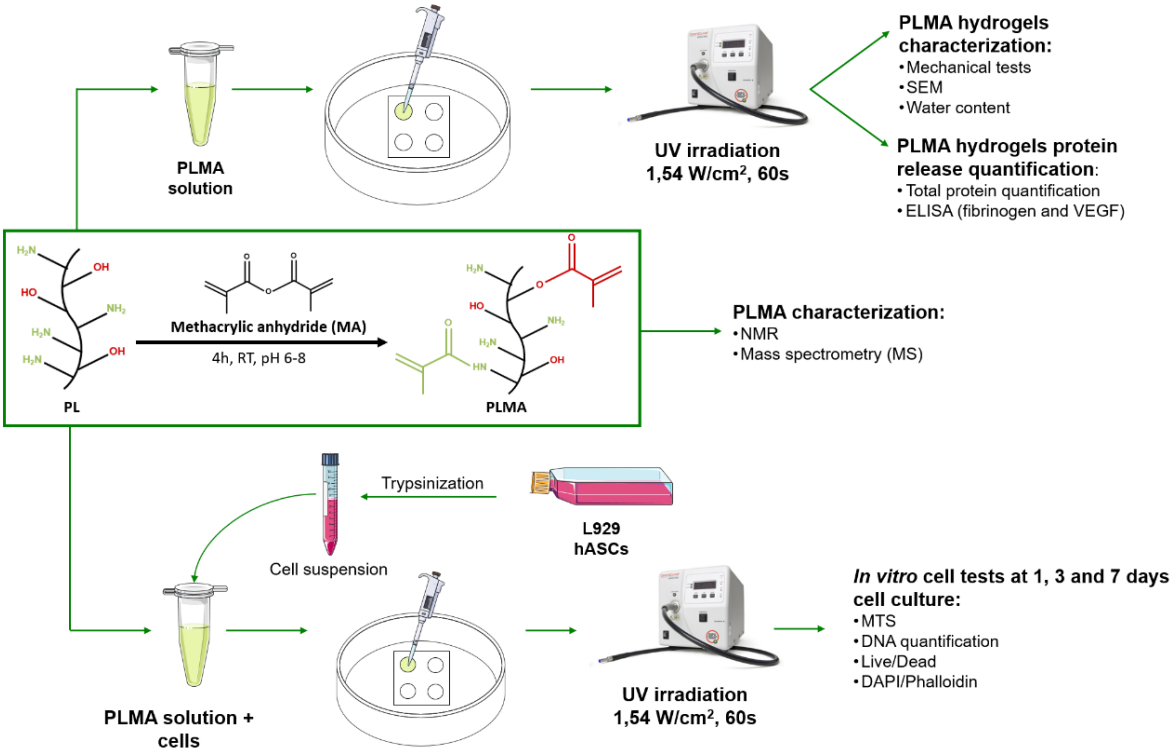

Figure III.2. Abstract scheme of the methods used in this work.

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Chapter IV*

Photopolymerizable customized platelet lysate hydrogels for 3D cell culture

* This chapter is based on the following publication:
Santos SC, Custódio CA and Mano JF. “Photopolymerizable customized platelet lysates hydrogels for 3D cell culture” (manuscript under preparation)

Photopolymerizable customized platelet lysates hydrogels for 3D cell culture

S. C. Santos[1], C. A. Custódio[1*], J. F. Mano[1*]

1- Department of Chemistry, CICECO, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal

* Corresponding author: catarinacustodio@ua.pt and jmano@ua.pt

Abstract

Regenerative medicine and tissue engineering (TE) aim the development of new tissue therapies capable of restore and maintain tissue function. Platelet-rich plasma (PRP) and human platelet lysates (PL) are attractive autologous sources of growth factors (GFs) and other molecules involved in the tissue regeneration process. PRP and PL have been mainly explored as a promising non-xenogenic serum supplement designed for the expansion of human cells that replaces fetal bovine serum. Recently, PRP/PL based scaffolds have been investigated as cell culture platforms for TE applications. Despite all the promising results, in most cases this type of materials have poor mechanical properties and also poor stability *in vitro*. In an attempt to overcome such limitations, we developed and characterize PL based hydrogels with tunable mechanical properties. PL were modified by addition of methacryloyl groups that by exposure to ultraviolet (UV) irradiation were capable of forming hydrogels. Our results showed that the achieved materials own increased mechanical properties that can be easily adjustable by changing methacryloyl PL (PLMA) concentration or PLMA modification degree. Additionally, PLMA hydrogels enabled sustained release of VEGF and fibrinogen for up to 250 hours and were resistant to protease degradation. Human derived stem cells and murine fibroblasts were successfully encapsulated in the hydrogels, exhibiting high cell viability, adhesion and proliferation. These results support the use of a PLMA hydrogel as a scaffold for stem cell culture and GFs release. We hypothesize that PLMA hydrogels may be an alternative to the gold standards for three-dimensional (3D) cell culture, namely Matrigel and collagen. The material here developed could have an autologous origin, being adequate to produce

customized robust matrices to be used as general platform for 3D cell culture with no risk of cross reactivity, immune reaction or disease transmission.

Introduction

Tissue engineering (TE) encompasses biological science and engineering in order to develop biological substitutes that can restore and maintain normal tissue function[1,2]. Although human body has the capacity to remodel and regenerate small defects, severe injuries or large defects are harder or even impossible to heal on its own[3]. Currently, therapies based on organ and tissue transplantation are widely used, however the lack of donors and also the severe immune complications are limitation of this strategy. So TE may be the answer to bypass these obstacles[1,4].

Hydrogels are soft three-dimensional (3D) insoluble hydrophilic network systems with high water content, that allow for an easy transport of oxygen, nutrients and cell metabolites. Due to its similarities with extracellular matrix (ECM) both structural and compositional level, hydrogels have received particular attention. Among other applications, hydrogels have been used as a platform for cell culture since they may promote cellular proliferation and survival[5–8].

Platelet-rich plasma (PRP), obtained from whole blood by a differential centrifugation process, is an autologous source of growth factors (GFs), cytokines and other protein[9–11]. Human platelet lysates (PL) are derivatives obtained from PRP by freeze/thaw cycles[12,13]. Previous works have reported the potential of PRP based materials for cell culture[14,15]. Moreover, PRP and PL have been used as an autologous material containing bioactive molecules, locally enhancing wound healing[16–19], bone growth and cartilage repair[20–23] and tissue sealing[24]. PRP and PL hydrogels have also been explored as 3D cell culture platforms and for tissue regeneration purposes. The major disadvantages of these hydrogels are their poor mechanical and poor stability *in vivo* and cell culture conditions which hinders its application. The main goal in this study was the development of hydrogels made of PL with increased mechanical properties and improved stability. Here we proposed the modification of PL by chemical conjugation with a photoresponsive group in order to achieve PL-based photopolymerizable materials with controlled mechanical properties. Such PL derived materials should provide a functional support for cell growth and interact with cells to control their function, guiding the process of tissue morphogenesis. Matrigel or

collagen matrices have shown to drive cellular self-organization and complex morphogenetic processes to result in sophisticated platforms for cell culture[25]. However, TE strategies using this type of materials goes against current animal-free approaches for cell culture. As an alternative to animal-based gels, synthetic polymer-based gels have also been developed to recreate native cell microenvironments[26]. Despite the design flexibility for specific applications, such synthetic systems are typically expensive and their synthesis can be time consuming, requiring often multiple steps. In the current era, striving toward personalized medicine and targeted therapy, creating the most appropriate material that closely mimics the *in vivo* microenvironment is one of the most important topics to address. Compared with using hydrogels, this method is advantageous in creating bioactive hydrogels without complicated synthesis for bioconjugation and no risk of cross reactivity, immune reaction or disease transmission.

We hypothesized that such blood-delivered hydrogels that could have an autologous origin, could be adequate to produce customized robust matrices rich in bioactive factor, adequate to be used as general platform for 3D cell cultures and in TE strategies.

Materials and methods

1. Synthesis of methacryloyl platelet lysates

Methacryloyl PL (PLMA) were synthesized by reaction with methacrylic anhydride 94% (MA) (Sigma-Aldrich, Germany). PL (STEMCELL Technologies, Canada) were thawed at 37ºC, to 10ml of this solution, either 100 or 300µL of MA was added, in order to synthesize PLMA100 (low-degree of modification) or PLMA300 (high-degree of modification), respectively. The reaction was performed under constant stirring during 4 hours at room temperature. pH was controlled and maintained between 6-8 by addition of 5M sodium hydroxide (NaOH) (AkzoNobel, USA) solution. During the first hour of reaction pH was constantly adjusted and then re-adjusted every 15 minutes. After 4 hours of reaction, the PLMA were purified by dialysis with Float-A-Lyzer G2 Dialysis Device 3.5-5 kDa (Spectrum, USA) against deionized water during 20-24h to remove the excess of MA. The PLMA solution was filtered with a 0.2 µm filter in order to sterilize it, frozen with liquid nitrogen, lyophilized and stored at 4ºC until further use.

2. PLMA characterization

2.1. ^{1}H NMR

NMR analysis were made in order to verify the functionalization of original PL. Solutions of 10 mg/mL of original PL and of each PLMA modification degree in dimethyl sulfoxide (DMSO) (Fisher Scientific, USA) were prepared and analyzed by ^{1}H NMR with 18 seconds relaxation delay and 300 scans. The ^{1}H NMR spectra were recorded on a Bruker AMX 300 spectrometer at 300.13 MHz.

2.2. Mass spectrometry

Mass spectrometric identification of proteins and modified proteins in PL and PLMA, respectively, was performed on a TripleTOF 6600 mass spectrometer after protein digestion with trypsin.

3. Hydrogel formation

Lyophilized PLMA were dissolved in a solution of 0.5% (w/v) 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone (Sigma-Aldrich) in PBS (Sigma-Aldrich) to final concentrations of 10% or 15% (w/v) PLMA. Hydrogels were made by pipetting the polymer solution to polydimethylsiloxane (PDMS) (Dow Corning, USA) molds with 3.5mm or 6mm diameter followed by ultraviolet (UV) irradiation (1.54 W/cm^{2}) during 60s.

4. PLMA hydrogels characterization

4.1. Mechanical properties

The mechanical behavior of PLMA hydrogels was characterized on the basis of compression tests employing a Universal Mechanical Testing Machine Shimadzu MMT-101N (Shimadzu Scientific Instruments, Kyoto, Japan) equipped with a load cell of 100N. To this end, both unidirectional and cyclic compression assays were performed at room temperature on as-prepared cylindrical hydrogels specimens with a diameter of 3.5 mm and height of 2 mm. The nominal stress was obtained by dividing the compressive load by the initial (uncompressed) cross-sectional area of the specimen. The Young's modulus was defined as the slope of the linear region of the strain/stress curve, corresponding to 0–25% strain. Ultimate stress and ultimate strain values were taken as the point where failure of the hydrogel occurred.

4.2. Scanning electron microscopy

The hydrogels were frozen with liquid nitrogen and then lyophilized. Samples were sputter-coated with gold and imaged via SEM using Ultra-high Resolution Analytical Scanning Electron Microscope HR-FESEM Hitachi SU-70 (Hitachi, Tokyo, Japan). SEM images were analyzed by Image J software for pore size measurement.

4.3. Water content

Hydrogel samples of PLMA100 and PLMA300 at 10% and 15% (w/v) were made in triplicate and immersed in 5 mL of PBS. Samples were incubated at room temperature during 2 hours. After 2 hours the wet weight (w_w) was measured and samples were frozen and lyophilized. After lyophilization, dry weight (w_d) was measured and compared with the initial wet weight. The water content was calculated according to following equation:

$$\text{Water content (\%)} = \frac{w_w - w_d}{w_w} \times 100$$

5. Quantification of protein and growth factors release

For the release assays, six samples of each PLMA100 at 10% and 15% (w/v) were made. Samples were added to falcons with 5 mL of PBS (Thermo Fischer Scientific, USA). The samples were incubated in a bath at 37ºC with constant agitation (60 rpm). At each time point, an aliquot of 600 µL was taken from each sample and 600 µL of fresh PBS added. Collected samples were stored at -20ºC. Total protein quantification was performed with Micro BCA Protein Assay Kit (Thermo Fisher Scientific). Quantification of specific proteins and GFs was performed by ELISA assay.

6. *In vitro* cell culture

6.1. Cell culture and encapsulation

The biological performance of PLMA hydrogels was assessed using the L929 Cell Line (European Collection of Authenticated Cell Cultures (ECACC)) and hASCs (ATCC). L929 cells were cultured in Dulbecco's Modified Eagle's Medium low glucose, supplemented with sodium bicarbonate, 10% FBS and 1% antibiotic/antimycotic. Cells were used between passages 3 and 25. hASCs were cultured in Minimum Essential Alpha Medium supplemented with sodium bicarbonate, 10% of FBS and 1% antibiotic/antimycotic. hASCs were used until passage 5. Cell suspensions were prepared by

trypsinization (trypsin/EDTA solution, Sigma-Aldrich). The cells were incorporated into the PLMA solutions to a final concentration of $2x10^6$ cells/mL in the case of L929 and $3x10^6$ cells/mL for hASCs. The hydrogels were prepared as described previously in section 3.

6.2. Live/Dead assay

At pre-determined time points, hydrogels were incubated in a solution of 2μL of Calcein AM 4mM solution in DMSO (Life Technologies, Thermo Fisher Scientific, USA) and 1μL of Propidium iodide (PI) (Thermo Fisher Scientific, USA) in 1000μL of PBS at 37ºC during 30 minutes. After washing with PBS, hydrogels were examined using a fluorescence microscope (Fluorescence Microscope Zeiss, Axio Imager 2, Zeiss, Germany).

6.3. Cell metabolic activity quantification

CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega, USA) was used. After each time point, hydrogels were washed with PBS and incubated in a solution of MTS reagent diluted 10x in PBS. Samples were then incubated overnight at 37ºC, protected from light. The quantification was achieved by measuring absorbance at 490nm. Triplicates were made for each sample and per culturing time.

6.4. Cell proliferation by DNA quantification

DNA quantification was performed using a Quant-iT PicoGreen dsDNA kit (Invitrogen, Thermo Fisher Scientific, USA). Briefly, at pre-determined time-points hydrogels were washed with PBS, incubated in sterile deionized water and frozen at -80ºC. In order to induce disruption of the cells inside the hydrogel, the samples were thawed at 37ºC and placed in an ultrasounds bath during approximately 30 minutes. Standards were prepared with concentrations ranging between 0 and 2 ug/mL. The plate was incubated for 10 min in the dark and fluorescence was measured using an excitation wavelength of 480 nm and an emission wavelength of 528 nm. Triplicates were made for each sample and per culturing time.

6.5. Cell morphology analysis

A DAPI/Phalloidin staining was made in order to access cell morphology in hydrogels. At pre-determined time-points hydrogels were washed with PBS and fixed with a 4% formaldehyde (Sigma-Aldrich) solution during at least 2 hours. For DAPI/Phalloidin

staining, a phalloidin solution (Flash Phalloidin™ Red 594, 300U, Biolegend, USA) was diluted 1:40 PBS and hydrogels were incubated at room temperature in phalloidin solution during 45 min. After PBS washes, a DAPI (DAPI (4',6-diamidino-2-phenylindole, dihydrochloride), Thermo Fisher Scientific) solution was diluted in 1:1000 PBS and hydrogels were incubated during 5 minutes with this solution also at room temperature. After washing with PBS, hydrogels were examined using a fluorescence microscope (Fluorescence Microscope Zeiss, Axio Imager 2, Zeiss, Germany).

6.6. Fabrication of PLMA microstructures

Lyophilized PLMA were dissolved in 0.5% (w/v) (w/v) 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone (Sigma-Aldrich) in PBS (Sigma-Aldrich) to a final concentration of 10% (w/v). L929 cells were incorporated into the PLMA solution to a final concentration of $2x10^6$ cells/mL. 50µL of this solution were poured onto a glass coverslip. Then, PDMS mold was brought into contact with the solution and pressed. The mold was exposed to UV irradiation (1.54 kW/cm^2) during 60s. Finally the PDMS mold was removed from the glass and the hydrogels were cultured during 7 days. A DAPI/Phalloidin staining was performed as described in 7.2.2.1 at 7 days of incubation and PLMA hydrogels were visualized using a fluorescence microscope (Fluorescence Microscope Zeiss, Axio Imager 2, Zeiss).

7. Statistical analysis

All data were subjected to statistical analysis and were reported as a mean ± standard deviation. Statistical differences between the analyzed groups were determine by unpaired *t* test.

Results and discussion

1. Synthesis and PLMA characterization

Human platelets are an autologous source of multiple proteins, GFs and cytokines that can be extracted by platelet lysis[27]. In this study, PL based hydrogels with tunable physical properties that can be used for cell encapsulation or as substrates for two-dimensional (2D) cell culture were synthesized for the first time. PLMA were synthesized

by reaction of PL with MA. Based on previously published works on the modification of gelatin and tropoelastin with methacrylate groups, we developed a protocol for the synthesis of PL proteins modified with methacrylate groups[8,28–32]. Different functional groups in the proteins present in PL are sensitive to chemical modifications as depicted in figure IV.1A.

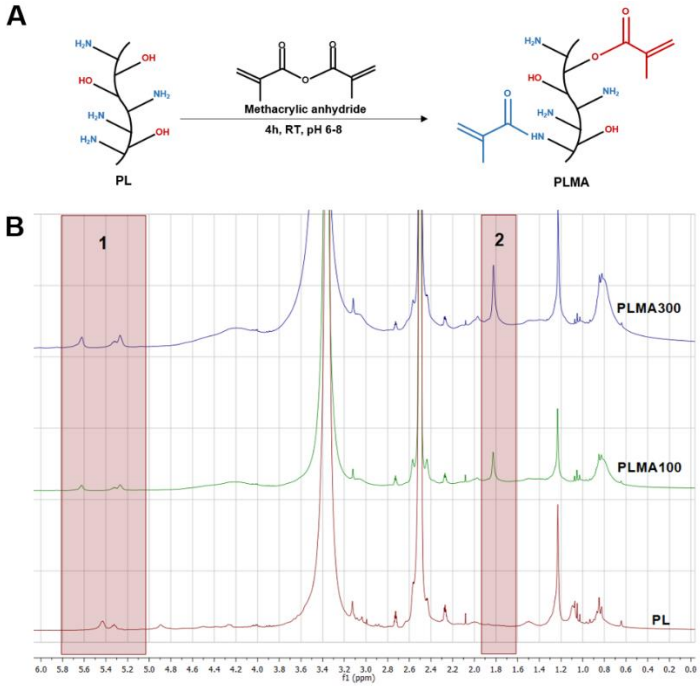

Figure IV.1. A) Methacrylate and methacrylamide groups by reaction of a protein/peptide with methacrylic anhydride. B) ^{1}H NMR spectra for PL, PLMA100 and PLMA300 with distinctive peaks characteristics of methacrylate groups: double bound methacrylate (1) and -CH_3 of methacrylate group (2).

In particular, the reactive functional groups are located on the side groups of amino acid residues, including hydroxyl groups, amino groups, and carboxylic acid substitutes. Different degrees of methacrylation could be obtained by varying the molar ratio of MA to PLs concentration. In this work two degrees of modification were achieved: PLMA100 and PLMA300. The degree of conversion with MA groups (i.e. degree of methacrylation) in biopolymers has conventionally been determined using ^{1}H NMR spectroscopy. Since PL is a mixture of proteins and GFs with complex compositions, it might not be feasible to detect and differentiate the resonance peaks from methacrylamide and methacrylate groups from ^{1}H NMR spectra. Nevertheless, ^{1}H NMR analysis to PLMA confirmed the methacrylation of PL by the appearance of distinctive peaks in the double bond region (δ 5.2 – 5.6 ppm) (figure IV.1B) and a sharp peak that corresponds to the -CH_3 of the methacrylate groups (δ ≈ 2 ppm) (figure IV.1B) on the modified PL spectra.

PL and PLMA samples were digested with trypsin in order to obtain small peptides. After protein digestion, mass spectrometry analysis was performed. Results show that human serum albumin is the most abundant protein in the PL mixture. Platelets, contain more than 1100 different proteins, with numerous post-translational modifications, resulting in over 1500 protein-based bioactive factors. Table IV.1. summarizes the main components found in the PL starting samples and MA modified samples. Results demonstrated that the main components of PL did not significantly change upon conjugation of protein PL with methacrylate groups.

	PL	PLMA100	PLMA300
1	Serum albumin	Serum albumin	Serum albumin
2	Apolipoprotein B-100	Serotransferrin	Complement C3
3	Complement C3	Complement C3	Serotransferrin
4	Alpha-2-macroglobulin	Alpha-2-macroglobulin	Alpha-2-macroglobulin
5	Serotransferrin	Keratin, type II cytoskeletal 1	Apolipoprotein B-100
6	Complement C4-B	Apolipoprotein B-100	Keratin, type II cytoskeletal 1
7	Keratin, type I cytoskeletal 10	Keratin, type I cytoskeletal 9	Alpha-1-antitrypsin
8	Immunoglobulin gamma-1 heavy chain	Apolipoprotein A-I	Keratin, type I cytoskeletal 10
9	Keratin, type II cytoskeletal 1	Keratin, type I cytoskeletal 10	Haptoglobin
10	Apolipoprotein A-I	Haptoglobin	Apolipoprotein A-I
11	Alpha-1-antitrypsin	Immunoglobulin gamma-1 heavy chain	Complement C4-B
12	Haptoglobin	Alpha-1-antitrypsin	Immunoglobulin gamma-1 heavy chain
13	Keratin, type II cytoskeletal 2 epidermal	Keratin, type II cytoskeletal 2 epidermal	Keratin, type II cytoskeletal 2 epidermal
14	Ceruloplasmin	Immunoglobulin heavy constant mu	Ceruloplasmin
15	Keratin, type I cytoskeletal 9	Immunoglobulin heavy constant alpha 1	Keratin, type I cytoskeletal 9
16	Apolipoprotein A-IV	Ceruloplasmin	Immunoglobulin heavy constant um
17	Hemopexin	Hemopexin	Hemopexin
18	Immunoglobulin mu heavy chain	Inter-alpha-trypsin inhibitor heavy chain H4	Alpha-1B-glycoprotein
19	Complement factor B	Keratin, type II cytoskeletal 5	Immunoglobulin heavy constant alpha 1
20	Complement factor H	Antithrombin-III	Inter-alpha-trypsin inhibitor heavy chain H4
21	Immunoglobulin heavy constant alpha 1	Keratin, type I cytoskeletal 14	Actin, cytoplasmic 2
22	Vitamin D-binding protein	Alpha-1B-glycoprotein	Immunoglobulin kappa light chain
23	Complement C5	Actin, cytoplasmic 2	Transthyretin
24	Actin, cytoplasmic 2	Immunoglobulin kappa light chain	Apolipoprotein A-IV
25	Alpha-1B-glycoprotein	Immunoglobulin heavy constant gamma 2	Inter-alpha-trypsin inhibitor heavy chain H2
26	Fibrinogen gamma chain	Complement factor B	Antithrombin-III
27	Fibrinogen alpha chain	Alpha-1-acid glycoprotein 1	Vitamin D-binding protein
28	Inter-alpha-trypsin inhibitor heavy chain H4	Vitronectin	Alpha-1-antichymotrypsin
29	Inter-alpha-trypsin inhibitor heavy chain H2	Inter-alpha-trypsin inhibitor heavy chain H1	Complement factor B
30	Immunoglobulin kappa light chain	Kininogen-1	Plasma protease C1 inhibitor
31	Alpha-1-antichymotrypsin	Fibrinogen gamma chain	Apolipoprotein E
32	Antithrombin-III	Alpha-1-antichymotrypsin	Immunoglobulin heavy constant gamma 2
33	Prothrombin	Complement C4-B	Vitronectin
34	Apolipoprotein E	Apolipoprotein A-IV	Fibrinogen gamma chain
35	Fibrinogen beta chain	Angiotensinogen	Angiotensinogen

Table IV.1. Protein content for PL, PLMA100 and PLMA300.

The degree of modification was also evaluated using mass spectrometry. As it was expected, mass spectrometry results for PLMA100 reveals less modified peptides with methacryloyl groups than in PLMA300 (Table IV.2) and most of these modification sites occur in serum albumin peptides (supporting information). Modification occurs in different residues but mainly in lysine (K) and glutamine (Q) residues, as also other researchers reported for gelatin and tropoelastin modification with this type of chemical conjugation[31].

Sample	Total peptide sequences	Modified peptide sequences	Degree of modification
PLMA100	2410	346	14%
PLMA300	3391	846	25%

Table IV.2. Mass spectrometry results for total number of identified peptides and also the number of modified peptides founded.

2. Hydrogel formation

Hydrogels were formed by photopolymerization upon exposure of a PLMA solution to UV irradiation. PLMA are water soluble and its low viscosity allow the preparation of PLMA solutions up to 15% (w/v). This characteristic is especially important in injectable hydrogels as this way its application is easier and use minimally invasive procedures. Another important characteristic of these hydrogels is the fact that the crosslinking occurs only in 60s UV exposure at quite low intensity, 1.54 W/cm^2. Figure IV.2 shows PLMA hydrogels obtained for each modification degree at 10% and 15% (w/v).

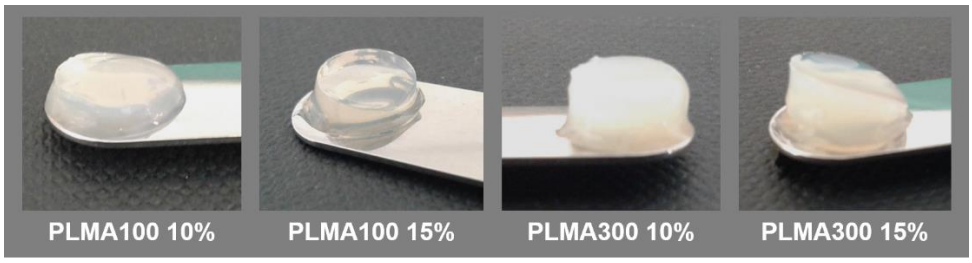

Figure IV.2. Crosslinked PLMA hydrogels formed from PLMA100 and PLMA300 at 10% and 15% (w/v).

3. PLMA hydrogels characterization

3.1. Mechanical tests

PL based gels formed upon activation of PL concentrates with calcium and thrombin have been used as materials for cell culture. Nevertheless, these hydrogels typically present poor mechanical properties and are difficult to handle. Moreover, they tend to degrade fast *in vivo*, unless some antifibrinolytic agent is used in order to slow down the degradation

process[33,34]. On the other side, material such as Matrigel and collagen, widely used as cell culture have issues with handling. To overcome these limitations, the hydrogels here proposed may be easily tuned to present enhanced stability and mechanical properties. We tested PLMA hydrogels mechanical properties and how they change by varying PLMA modification degree and concentration. Hydrogels were formed by addition of a photoinitiator to the PLMA solution followed by photopolymerization with UV irradiation. Mechanical assays were performed in hydrogels made from PLMA100 and PLMA300, prepared upon UV irradiation (1.54 W/cm^2) during 60s. For both PLMA degrees of modification is possible to obtain robust hydrogels at 10% and 15% (w/v). Figure IV.3A shows the results obtained for compressive stress-strain of PLMA hydrogels.

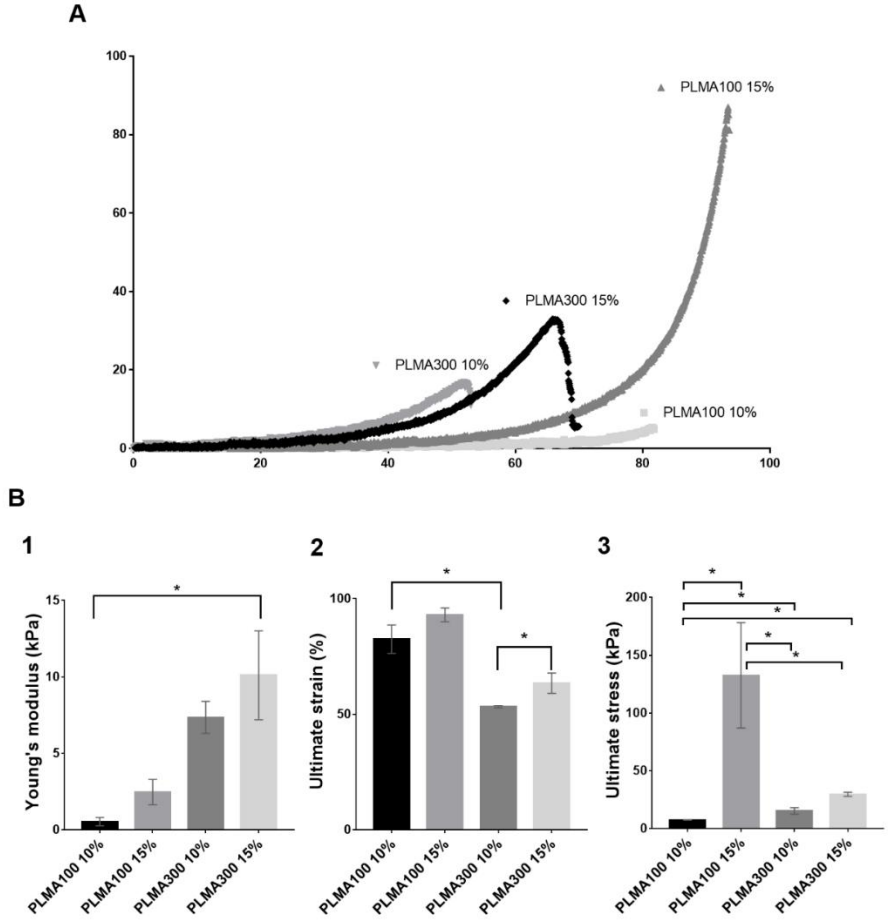

Figure IV.3. A) Representative compressive stress-strain curves for PLMA100 and PLMA300 hydrogels at 10% and 15% (w/v). B) Young's modulus (1), ultimate strain (2) and ultimate stress (3) obtained for PLMA100 and PLMA300 at 10% and 15% (w/v). Statistical analysis through unpaired *t* test showed significant differences (*$p < 0.05$) between the analyzed groups.

The data shown in figure IV.3B-1 shows that Young's modulus increases with the increase in the PLMA concentration. Likewise, an increase in the degree of modification of PL, corresponds to an increase in stiffness for the same concentration of polymer. As it can be seen, for PLMA100 Young's modulus at 10% and 15% (w/v) is lower when compared with the same concentrations of PLMA300 hydrogels. Figure IV.3B-2 shows the ultimate strain for PLMA hydrogels and the influence of polymer concentration and degree of modification on these values. The ultimate strain is higher in PLMA 15% (w/v) and also higher in PLMA100 modification compared with PLMA300. PLMA300 have less resistance to fracture compared to PLMA100 modification hydrogels. This may be due to the higher degree of methacrylation, which increases the crosslinking density of hydrogels and also its stiffness. Figure IV.3B-3 shows the ultimate stress for each concentration of two different modification degrees. An increase in the ultimate stress from the 10% (w/v) to 15% (w/v) hydrogels is evident. It is important to point out that PLMA hydrogels are formed rapidly within 30 - 60 seconds upon UV irradiation, which is shorter than the typical time (~ 20min - 1h) required to crosslink PRP-based gels.

Evaluate mechanical properties of hydrogels is an important step in their characterization. The mechanical properties play a key role in the stability of the hydrogel in culture and in cellular mechanotransduction, this way affecting cellular spreading, migration and also differentiation. Provide the ideal features to support cell adhesion and proliferation are important characteristics of 3D cell culture platforms[35–37]. The PLMA hydrogels have unique structural properties that allow them to behave as a viscoelastic solid with improved mechanical properties compared to PRP based gels so far reported.

Mechanical properties of PLMA hydrogels may be tuned in the range of 0.5 – 10 kPa. Therefore, PLMA hydrogels may be used to mimic some native tissues stiffness: brain tissue (elasticity 0.1-1 kPa)[36,38–40] and striated muscle (elasticity 8-17 kPa)[38].

3.2. Physical properties of PLMA hydrogels

Hydrogels porosity influences nutrient flux throughout the matrix and is related with hydrogel swelling and mechanical properties[35]. Small molecular porosity is correlated with lower swelling ratio and higher modulus[35,41,42]. Molecular porosity and mechanical properties of hydrogels are important characteristics that have to be taken into account due to its influence in cell behavior. Penetration of cells into hydrogels, as well as migration, proliferation and exchange of oxygen, nutrients and waste materials are process influenced

by the hydrogel stiffness[43]. In order to explore PLMA hydrogels microstructure, SEM analysis of dried PLMA gels were performed. PLMA hydrogels have a porous network influenced by PLMA concentration as showed in figure IV.4. For lower (10% w/v) PLMA concentration, hydrogels have larger porous than in higher (15% w/v) PLMA concentrations. Also methacryloyl modification degree influence these porous network, as it can be seen in figure IV.4, PLMA300 hydrogels show a decrease in pore size, especially for PLMA300 15% (w/v).

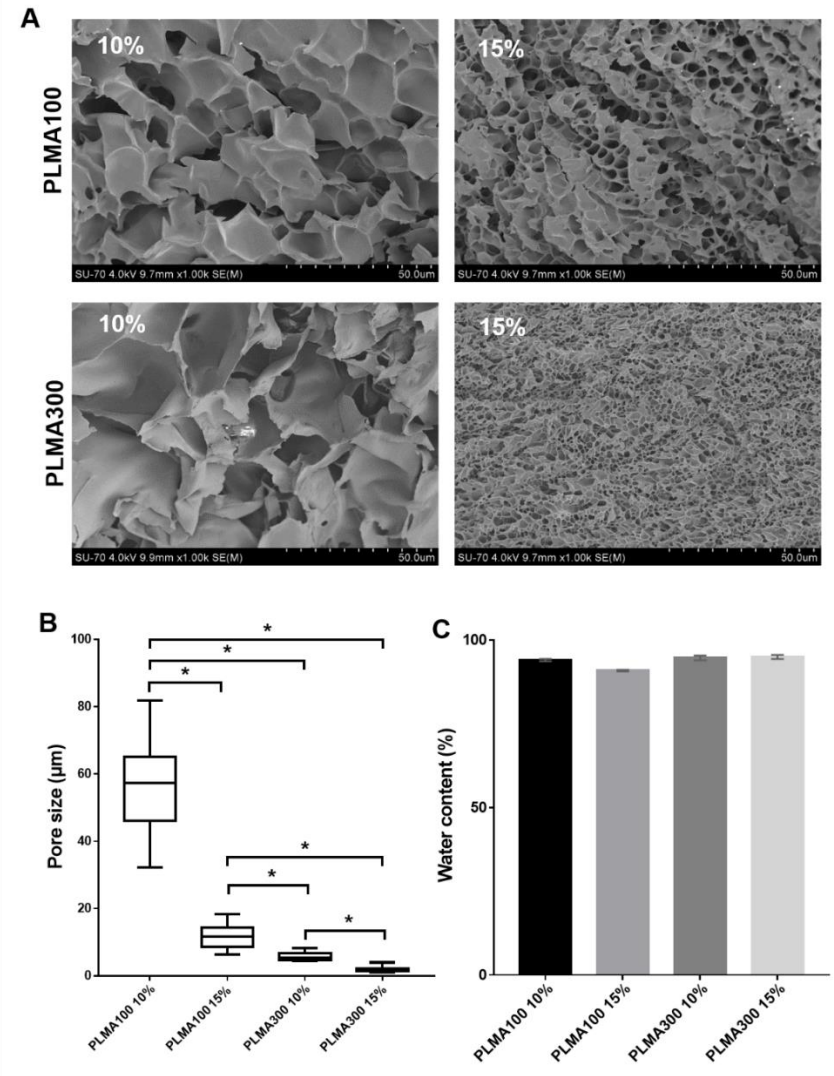

Figure IV.4. A) Representative cross-section SEM images of PLMA100 and PLMA300 hydrogels at 10% and 15% (w/v). B) Pore size values obtained for PLMA100 and PLMA300 at 10% and 15% (w/v) hydrogels. C) Water content for PLMA100 and PLMA300 hydrogels at 10% and 15% (w/v). Statistical analysis through unpaired *t* test showed significant differences (*$p < 0.05$) between the analyzed groups.

Molecular porosity of PLMA hydrogels is not very different of other protein based hydrogels like gelatin methacryloyl[43] and it can be tuned by changing some hydrogel fabrication parameters, which does not occur for other hydrogels like tropoelastin methacrylate based hydrogels[32] where pore size is not very different for each low modification degree concentration.

The water content of PLMA hydrogels was also evaluated. Results shown that this parameter is not significantly different between all the studied conditions. In general, PLMA hydrogels have 90% of water content (Figure IV.4C).

4. *In vitro* cell culture studies

L929 mouse fibroblasts were used in a first screening of the biological performance of the PLMA hydrogels. Cells encapsulated in PLMA100 hydrogels at 10% (w/v) were cultured during 7 days and at determined time points, namely 1, 3 and 7 days, analysis to cell morphology, viability and proliferation were performed. Figure IV.5 shows that cells are well distributed within the hydrogel, exhibiting high viability at 1 day and also after 7 days. DAPI/Phalloidin staining was also performed at days 3 and 7 in order to see cell morphology inside the PLMA hydrogels (figure IV.5). The L929 cells adhered, spread and proliferated within the PLMA hydrogel.

In a second approach, hASCs were used in order to test the potential of PLMA hydrogels to support the culture of human derived stem cells. In a first approach, hydrogels made of PLMA100 at 10% (w/v) were used for hASCs encapsulation likewise for L929 culture. However, the results revealed that these hydrogels were not capable to promote hASCs adhesion and proliferation. Based on literature reports that suggest stiffer hydrogels for increased hASCs adhesion and proliferation[44,45], PLMA100 hydrogels at a 15% (w/v) concentration were tested. Live/Dead assay reveal that PLMA hydrogels greatly support cell viability up to 7 days in culture (figure IV.5A). Cell morphology was assessed by DAPI/Phalloidin staining at 3 and 7 days of culture (Figure IV.5B and 5C). After 3 days in culture, cells readily elongated in PLMA hydrogels, after 7 days in culture cells are perfectly elongated and migrated, forming interconnected networks with neighboring cells.

Photopolymerizable hydrogels permit to fix the shape of the solution precursor using light. In order to fabricate microstructures of PLMA hydrogels, L929 cells were encapsulated in a PLMA100 10% (w/v) hydrogel (figure IV.5D). Obtained microstructures have approximately 800µm and L929 cell are able to proliferate inside these microgels.

These results support the idea that PLMA hydrogels can be used as individual microstructures for biological tests or as hybrid units that can be then assembled into bigger structures.

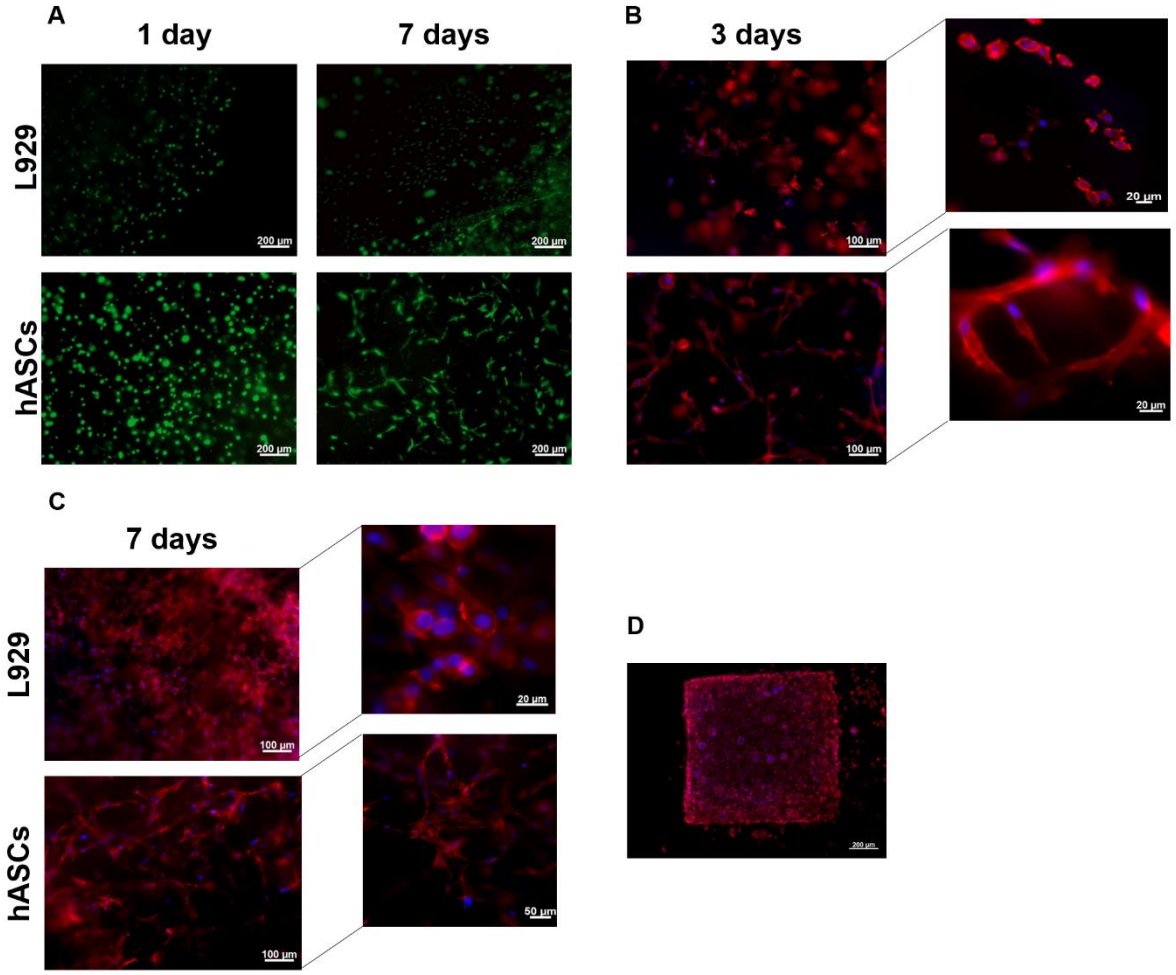

Figure IV.5. Representative fluorescence images for: A) L929 and hASCs live/dead at 1 and 7 days of culture. B and C) L929 and hASCs DAPI/Phalloidin staining at 3 and 7 days of cell culture. (D) DAPI/Phalloidin staining for PLMA microstructures of encapsulated L929 at 7 days of culture.

Cell viability and cell proliferation assays were performed by MTS test and DNA quantification, respectively (Figure IV.6). L929 as well as hASCs are able to proliferate inside PLMA hydrogels as it can be seen by the increasing in DNA quantity and also by the results achieved for viability tests. These results are in agreement with previously described results for live/dead assay and DAPI/Phalloidin staining, which demonstrated cell spreading and proliferation within PLMA hydrogels.

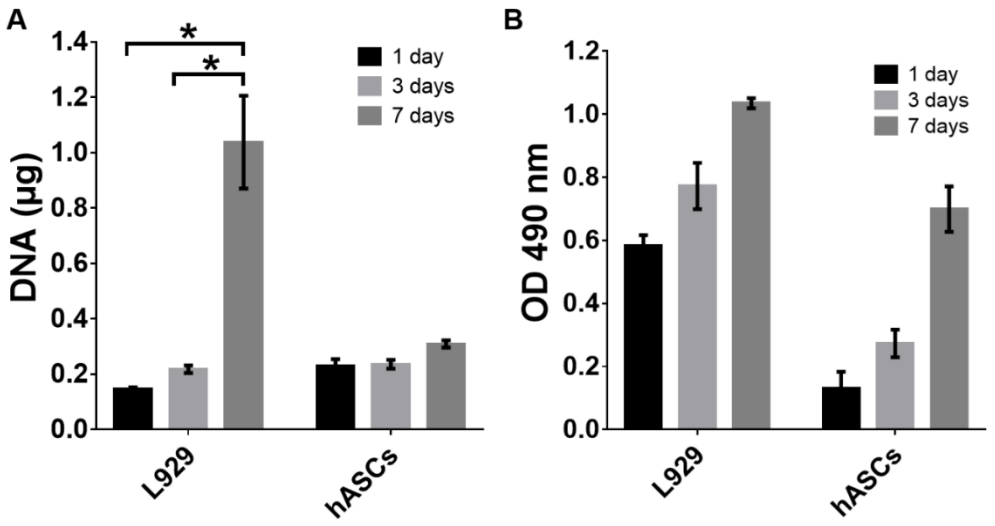

Figure IV.6. DNA (A) and MTS (B) results for L929 and hASCs at 1 day, 3 and 7 days of cell culture. Statistical analysis through unpaired *t* test showed significant differences (*p < 0.05) between the analyzed groups.

The obtained results showed that PLMA hydrogels can be used as a platform for cell culture. Cells maintain their viability for at least 7 days and are able to proliferate inside the hydrogels. However, depending on cell type, a specific stiffness of PLMA hydrogels is required. hASCs adhesion was poor on PLMA100 10% (w/v) hydrogels and increased with an increase of PLMA to 15%, that is associated with increased stiffness. The stiffness of the hydrogel is important in cell adhesion and proliferation process and has to be tuned in order to obtain the expected results[38,44,46,47]. Both cell types readily bound to, proliferated, elongated and migrated when encapsulated on PLMA hydrogels. The relatively soft nature of PLMA gel likely contributes to the favorable cell invasion and migration. Such results are quite promising, thus far, most of the hydrogels used in cell encapsulation require often conjugation with specific bioactive molecules[34,42,48,49]. This may be time consuming and costly. The intrinsic ability of PLMA hydrogels to support cell culture is comparable to that of Matrigel or collagen. Matrigel or collagen matrices have shown to drive cellular self-organization and complex morphogenetic processes to result in sophisticated *in vitro* disease models[26,50]. As an alternative to animal-based gels, synthetic polymer-based gels have also been developed to recreate native cell microenvironments. However, they are typically expensive and their synthesis can be time consuming, requiring often multiple steps. In addition, nowadays there is an effort to switch to animal-free models. We further envisage

that PLMA hydrogels may be an alternative to these animal derived materials for 3D cell culture.

5. Protein released from PLMA hydrogels

PLMA hydrogels here described are a pool of proteins, GFs and other molecules that are essential for cell growth and proliferation. We predicted that a fraction of this complex mixture of proteins will be loosely bounded to the hydrogel after the photocrosslinking step. For this reason, protein and GFs release assays were performed in order to know the profile release from PLMA hydrogels. Figure IV.7A shows the total protein release profile from the gels used for *in vitro* cell encapsulation assays, PLMA100 at 10% and 15% (w/v). Protein release profile of PLMA hydrogels shows an overall sustained release. In the first 24 hours of the assay, protein release is quite fast but between 24 hours and approximately 240 hours protein release is well controlled. Using autologous source of GFs instead of using recombinant GFs, which are very expensive and have a short half-life, has been proposed[51–53]. PLMA hydrogels are a pool of these GFs and they can be potentially used as a platform for the release of autologous GFs and bioactive proteins involved in cell recruitment, growth and proliferation processes. The vascular endothelial growth factor (VEGF) has the potential to promote differentiation of common stem cells to angiogenic cells and has been long recognized as an important and necessary step during tissue repair. On the other hand, fibrinogen has been recognized as a main component in PRP and is a key element in the previous reported PRP based gels. Thus, ELISA assay was performed to investigate the release profile of both VEGF and fibrinogen from the PLMA hydrogels. As well as in total quantification, the release of fibrinogen and VEGF occurs in a sustained manner as figure IV.7B shows.

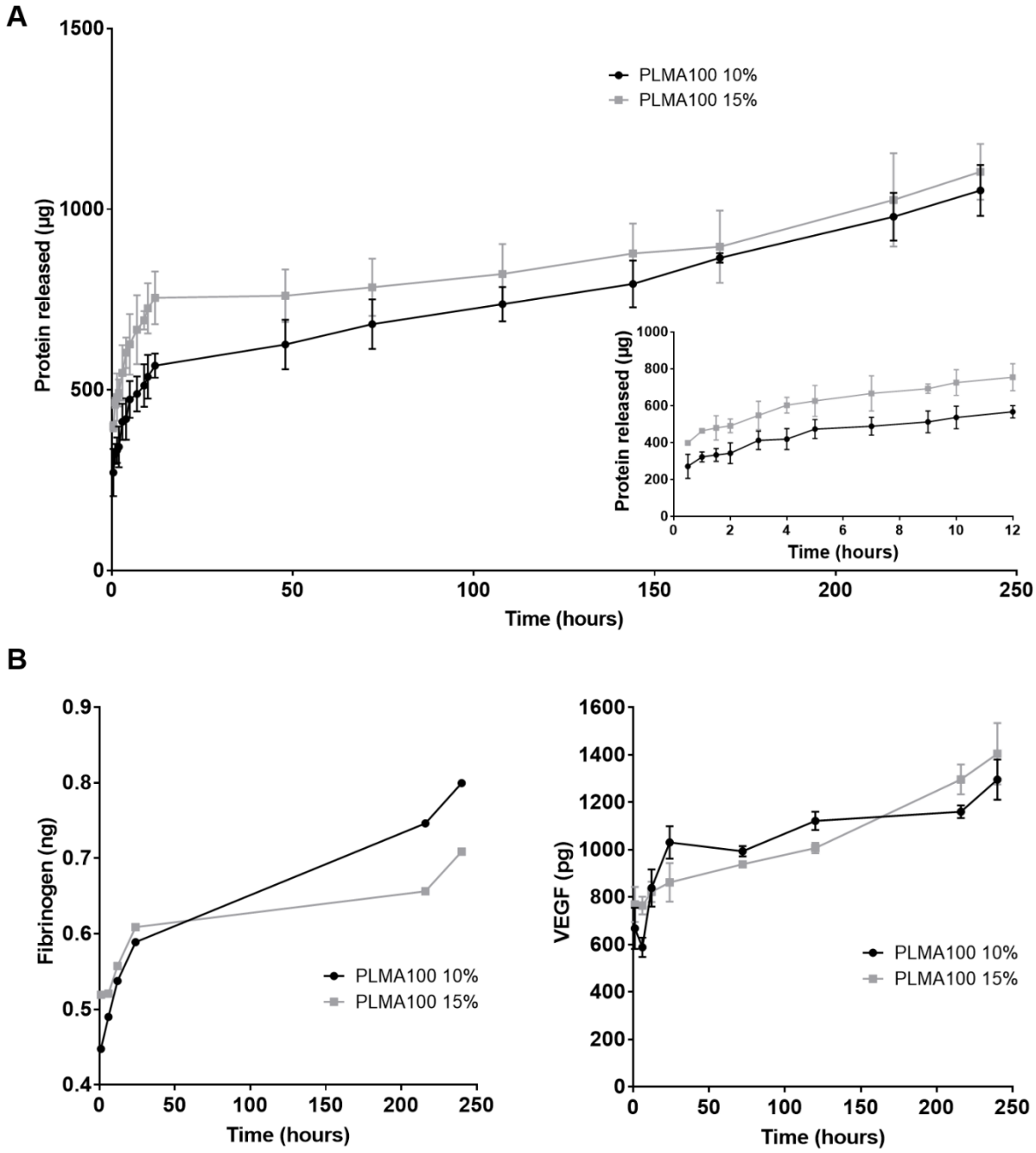

Figure IV.7. Total protein release quantification (A) and ELISA quantification of fibrinogen and VEGF (B) for PLMA100 10% and 15% (w/v).

Conclusions

PRP and PL are attractive sources of GFs that have been widely studied for TE applications. Previous studies found in the literature concerning the use of hydrogels based on PRP, mostly report the activation with bovine thrombin and crosslinking with calcium. Despite the potential of those systems, the limited mechanical properties, and low stability *in vitro* results in an ineffective strategy. Herein we reported for the first time, a direct modification of PL proteins with a photoresponsive group. Such modification allow the production of PL derived photopolymerized hydrogels with tunable mechanical properties. The PLMA based hydrogels here proposed not only have increased mechanical properties but also higher stability *in vitro* when compared to PRP/PL based reported materials. PLMA based hydrogels support the growth of encapsulated cells. Our results suggest that such type of gels may be an alternative to collagen and Matrigel, the gold standards to provide 3D cell cultures for a wide range of cell types. A foremost advantage of PLMA based gels is their human origin, following the current animal-free approaches and autologous strategies for cell culture and biomedical applications. The bioactivity of the modified proteins should be understood and will be performed in future studies. Recognizing the unprecedented potential of the PLMA gels to support 3D cell culture, we hypothesize that PLMA based gels may be used as cell culture platforms avoiding the use of animal derived supplements.

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Chapter V

Conclusions and future perspectives

Conclusions and future perspectives

Regenerative medicine and tissue engineering (TE) are emerging areas aiming to develop new strategies for tissue healing and regenerative process. Platelets are a natural reservoir of GFs and are activated at sites of tissue injury, where they promote tissue repair, including revascularization. PRP injections have become popular, particularly with treatments for musculoskeletal injuries. In addition, PRP and its derivatives like platelet lysates (PL) have recently emerged as promising candidates for replacing animal serum in cell culture.

PRP also contains extracellular matrix (ECM) precursors, like fibrinogen, offering a fully human option to engineer a biodegradable scaffold for cell encapsulation and 3D cell culture. In fact, PRP based gels have been widely explored as implantable materials for tissue regeneration, still these materials have poor mechanical properties and poor stability *in vitro* and *in vivo*. In this contribution, the advances in the applications of plasma derived materials are critically reviewed and their shortcomings are pointed out in chapter 3 of this thesis. In addition, we highlight the limitations of the currently PRP based hydrogels as 3D matrices for cell culture.

The major goal of this thesis was the development of a novel process for making a crosslinked blood plasma derived hydrogel, which shows increased stability compared to PRP based hydrogels of the art. In chapter 4 of this thesis the development and characterization of PRP/PL based hydrogel which are bioactive and have tunable mechanical properties are reported. More particularly, the present work relates to the modification of PL proteins with a methacryloyl group (PLMA) that allows further chemical crosslinking upon UV light. PLMA hydrogels here proposed have increased mechanical properties and higher stability *in vitro* when compared to PRP/PL reported in the literature. It was here demonstrated that the mechanical properties and microstructure of PLMA gels can be easily tuned by changing PL degree of modification and material concentration. Biological studies, reveal that PLMA gels support cell growth for over 7 days. Remarkably, since day 1 the encapsulated human cells could also perform important biological processes such as growth and migration. The sustained release of proteins and growth factors was also observed. Future studies will include the culture of human derived cells within the PLMA gels without animal serum as supplement. A main objective of the strategy here proposed it is the exclusion of animal derived components in the *in vitro* assays to reduce the potential

contamination problems. The final goal of this project is the use of complete autologous materials and cells from a patient, this open up new possibilities for personalized medicine and thus targeted therapeutic approaches.

This is the first study to demonstrate that chemical modification of proteins from PL, can be used to fabricate customizable hydrogels for 3D cell culture. Results shown that PLMA hydrogels provide the necessary structural and biochemical support for cell growth, and particularly suitable for tissue engineering or development of microtissues for drug screening.

Supporting information

PLMA100			
Names	**Sequence**	**Modifications**	**dMass**
Serum albumin	AFKAWAVAR	Methacrylic anhydride(K)@3	-0,0053044
	FKDLGEENFK	Methacrylic anhydride(K)@2	0,00236207
	KQTALVELVK	Methacrylic anhydride(K)@1	-0,0121997
	KYLYEIAR	Methacrylic anhydride(K)@1	-0,00769923
	LAKTYETTLEK	Methacrylic anhydride(K)@3	-0,00462136
	QIKKQTALVELVK	Methacrylic anhydride(K)@4	0,022857601
	YTKKVPQVSTPTLVEVSR	Methacrylic anhydride(K)@3; Methacrylic anhydride(K)@4	-0,001706
	TKEQLKAVMDDFAAFVEK	Methacrylic anhydride(K)@2; Methacrylic anhydride(Q)@4; Methacrylic anhydride(K)@6	-0,0118432
	QTALVELVKHKPK	Methacrylic anhydride(K)@9; Methacrylic anhydride(K)@11	0,00915571
	ADDKETCFAEEGKK	Methacrylic anhydride(K)@13	0,0195789
	ADDKETCFAEEGKK	Methacrylic anhydride(K)@14	0,0195789
	ADDKETCFAEEGKK	Methacrylic anhydride@N-term	-0,0115307
	AEFAEVSK	Methacrylic anhydride@N-term	-0,01199
	AVMDDFAAFVEK	Methacrylic anhydride@N-term	-0,0126826
	CCKADDKETCFAEEGKK	Methacrylic anhydride(K)@3	-0,028849101
	DAHKSEVAHR	Methacrylic anhydride@N-term; Methacrylic anhydride(K)@4	8,74E-05
	DDNPNLPR	Methacrylic anhydride@N-term	-0,0175192
	EQLKAVMDDFAAFVEK	Methacrylic anhydride(K)@4	0,00211765
	FDEFKPLVEEPQNLIK	Methacrylic anhydride(K)@5	0,0269637
	FKAWAVAR	Methacrylic anhydride(K)@2	-0,0150526
	FKDLGEENFK	Methacrylic anhydride(K)@2	0,00236207
	FPKAEFAEVSK	Methacrylic anhydride(K)@3	-0,0044001
	FQNALLVR	Methacrylic anhydride(Q)@2	-0,0132629
	FQNALLVR	Methacrylic anhydride@N-term	-0,0123888
	FYAPELLFFAKR	Methacrylic anhydride(R)@12	0,0245356
	HPYFYAPELLFFAK	Methacrylic anhydride@N-term	-0,0144739
	HPYFYAPELLFFAK	Methacrylic anhydride(H)@1	-0,0144739
	HPYFYAPELLFFAKR	Methacrylic anhydride(R)@15	0,0223991
	KQTALVELVK	Methacrylic anhydride(K)@1	-0,0121997
	KQTALVELVK	Methacrylic anhydride(Q)@2	0,0115714
	KVPQVSTPTLVEVSR	Methacrylic anhydride(K)@1	-0,0139793
	KVPQVSTPTLVEVSR	Methacrylic anhydride(Q)@4	-0,049004599

	KVPQVSTPTLVEVSR	Methacrylic anhydride(K)@1; Methacrylic anhydride(Q)@4	0,000517534
	KYLYEIAR	Methacrylic anhydride(K)@1	-0,00824722
	LAKTYETTLEK	Methacrylic anhydride(K)@3	-0,00462136
	LAKTYETTLEK	Methacrylic anhydride@N-term	0,020749699
	LCTVATLR	Methacrylic anhydride@N-term	-0,0128611
	LDELRDEGK	Methacrylic anhydride@N-term	0,00616255
	LDELRDEGKASSAK	Methacrylic anhydride(R)@5; Methacrylic anhydride(K)@9	0,00113728
	LDELRDEGKASSAK	Methacrylic anhydride(R)@5	0,0274675
	LDELRDEGKASSAKQR	Methacrylic anhydride(K)@9; Methacrylic anhydride(K)@14	0,0022549
	LDELRDEGKASSAKQR	Methacrylic anhydride(K)@14	-0,021606401
	LDELRDEGKASSAKQR	Methacrylic anhydride(K)@14; Methacrylic anhydride(Q)@15	0,00276091
	LVAASQAALGL	Methacrylic anhydride@N-term	-0,0116827
	LVNEVTEFAK	Methacrylic anhydride@N-term	-0,011087
	LVRPEVDVMCTAFHDNEETFLKK	Methacrylic anhydride(K)@22	-0,017450901
	QIKKQTALVELVK	Methacrylic anhydride(Q)@1; Methacrylic anhydride(K)@4	0,000304357
	QIKKQTALVELVK	Methacrylic anhydride(K)@4	0,021931199
	QNCELFEQLGEYK	Methacrylic anhydride(Q)@1	-0,0132572
	QNCELFEQLGEYK	Methacrylic anhydride@N-term	-0,0132572
	QNCELFEQLGEYK	Methacrylic anhydride(N)@2	-0,0123137
	RHPDYSVVLLLR	Methacrylic anhydride(H)@2	0,0065927
	RYKAAFTECCQAADK	Methacrylic anhydride(R)@1	-0,00166275
	TKCCTESLVNR	Methacrylic anhydride(K)@2	0,021412401
	TKEQLKAVMDDFAAFVEK	Methacrylic anhydride@N-term; Methacrylic anhydride(K)@2; Methacrylic anhydride(K)@6	-0,0118432
	TKEQLKAVMDDFAAFVEK	Methacrylic anhydride(K)@2; Methacrylic anhydride(Q)@4; Methacrylic anhydride(K)@6	-0,0123628
	VFDEFKPLVEEPQNLIK	Methacrylic anhydride@N-term	-0,016495099
	VGSKCCKHPEAK	Methacrylic anhydride(K)@7	-0,0109704
	VGSKCCKHPEAK	Methacrylic anhydride(K)@4	-0,0109704
	VNEVTEFAK	Methacrylic anhydride(N)@2	0,0131231
	VPQVSTPTLVEVSR	Methacrylic anhydride@N-term; Methacrylic anhydride(Q)@3	0,0154781
	VTKCCTESLVNR	Methacrylic anhydride(K)@3	-0,0113639
	YKAAFTECCQAADK	Methacrylic anhydride@N-term	0,027179301
	YTKKVPQVSTPTLVEVSR	Methacrylic anhydride(K)@3; Methacrylic anhydride(K)@4	-0,001706
Serotransferrin OS=Homo sapiens GN=TF PE=1 SV=3	KASYLDCIR	Methacrylic anhydride(K)@1	-0,00667122
	CLKDGAGDVAFVK	Methacrylic anhydride(K)@3	-0,024202799
	KCSTSSLLEACTFR	Methacrylic anhydride(K)@1	-0,00331561

Apolipoprotein A-I	QKVEPLRAELQEGAR	Methacrylic anhydride(K)@2	-0,000459271
	KWQEEMELYR	Methacrylic anhydride(W)@2	0,028870599
Immunoglobulin gamma-1 heavy chain	GPSVFPLAPSSKSTSGGTAALGCLVK	Methacrylic anhydride(K)@12	-0,00536033
	FNWYVDGVEVHNAK	Methacrylic anhydride@N-term	-0,016043199
	FNWYVDGVEVHNAK	Methacrylic anhydride(W)@3	-0,016043199
Immunoglobulin heavy constant alpha 1	DASGVTFTWTPSSGKSAVQGPPER	Methacrylic anhydride(K)@15	-0,00264049
	VAAEDWKK	Methacrylic anhydride(K)@7	-0,0252777
	WLQGSQELPR	Methacrylic anhydride@N-term	-0,0100065
	WLQGSQELPR	Methacrylic anhydride(W)@1	-0,0100065
Immunoglobulin heavy constant gamma 2	FNWYVDGVEVHNAK	Methacrylic anhydride(W)@3	-0,016043199
	FNWYVDGVEVHNAK	Methacrylic anhydride@N-term	-0,016043199
Immunoglobulin lambda constant 2	ADSSPVKAGVETTTPSK	Methacrylic anhydride(K)@7	-0,00450191
	YAASSYLSLTPEQWKSHR	Methacrylic anhydride(K)@15	-0,0234867
Immunoglobulin alpha-2 heavy chain	TTVTVSSASPTSPK	Methacrylic anhydride@N-term	0,025227699
	VAAEDWKK	Methacrylic anhydride(K)@7	-0,0252777
	WLQGSQELPR	Methacrylic anhydride(W)@1	-0,0100065
	WLQGSQELPR	Methacrylic anhydride@N-term	-0,0100065
Immunoglobulin lambda-1 light chain; Immunoglobulin lambda-like polypeptide 5	YAASSYLSLTPEQWKSHR	Methacrylic anhydride(K)@15	-0,0234867
Immunoglobulin heavy constant gamma 4	FNWYVDGVEVHNAK	Methacrylic anhydride(W)@3	-0,016043199
	FNWYVDGVEVHNAK	Methacrylic anhydride@N-term	-0,016043199
Glutamate receptor ionotropic, NMDA 1	QNVSLSILK	Methacrylic anhydride(K)@9	-0,0131732
Purine nucleoside phosphorylase	VIMDYESLEK	Methacrylic anhydride(K)@10	-0,0130048

Table 1. MA modified peptides and correlated proteins for PLMA100 modification.

PLMA300			
Names	**Sequence**	**Modifications**	**dMass**
Serum albumin	AACLLPKLDELR	Methacrylic anhydride(K)@7	-0,00233865
	KYLYEIAR	Methacrylic anhydride(K)@1	-0,00370284
	LDELRDEGKASSAKQR	Methacrylic anhydride(K)@9; Methacrylic anhydride(K)@14	0,00298238
	LECADDRADLAKYICENQDSISSK	Methacrylic anhydride(K)@12	-0,0217715
	LKCASLQKFGER	Methacrylic anhydride(K)@2; Methacrylic anhydride(K)@8	-0,00306525
	LVNEVTEFAKTCVADESAENCDK	Methacrylic anhydride(K)@10	-0,0105955
	NECFLQHKDDNPNLPR	Methacrylic anhydride(K)@8	0,00180468
	SLHTLFGDKLCTVATLR	Methacrylic anhydride(K)@9	-0,0040148
	TKEQLKAVMDDFAAFVEK	Methacrylic anhydride(K)@2; Methacrylic anhydride(Q)@4; Methacrylic anhydride(K)@6	-0,00493928
	VGSKCCKHPEAK	Methacrylic anhydride(K)@7	-0,025557499
	VTKCCTESLVNR	Methacrylic anhydride(K)@3	-0,00730587
	YKAAFTECCQAADKAACLLPK	Methacrylic anhydride(K)@2; Methacrylic anhydride(K)@14	-0,0156286
	ETYGEMADCCAKQEPERNECFLQHKDDNPNLPR	Methacrylic anhydride(K)@12	0,0286284
	CCKADDKETCFAEEGKK	Methacrylic anhydride(K)@3	-0,0225899
	YTKKVPQVSTPTLVEVSR	Methacrylic anhydride(K)@3	0,0223688
	ATKEQLK	Methacrylic anhydride(K)@3	-0,00475801
	LAKTYETTLEKCCAAADPHECYAK	Methacrylic anhydride(K)@3; Methacrylic anhydride(K)@11	0,00145459
	QIKKQTALVELVK	Methacrylic anhydride(K)@3; Methacrylic anhydride(K)@4	-0,00038923
	PKATKEQLKAVMDDFAAFVEK	Methacrylic anhydride(K)@2; Methacrylic anhydride(K)@5; Methacrylic anhydride(K)@9	-0,0348919
	FPKAEFAEVSKLVTDLTK	Methacrylic anhydride(K)@3; Methacrylic anhydride(K)@11	0,000838106
	FYAPELLFFAKR	Methacrylic anhydride(K)@11	-0,023745099
	DHVKLVNEVTEFAK	Methacrylic anhydride(K)@4	0,0264995
	KEQLKAVMDDFAAFVEK	Methacrylic anhydride(K)@5	0,000375254
	ADDKETCFAEEGK	Methacrylic anhydride(K)@4	-0,0033294
	ADDKETCFAEEGKK	Methacrylic anhydride(K)@14	-0,0145434
	ADDKETCFAEEGKK	Methacrylic anhydride(K)@13	-0,0145434
	AEFAEVSK	Methacrylic anhydride@N-term	-0,0124118
	AEFAEVSKLVTDLTK	Methacrylic anhydride(K)@8	-0,0150789
	AFKAWAVAR	Methacrylic anhydride(K)@3	-0,00939401
	ATKEQLKAVMDDFAAFVEK	Methacrylic anhydride(K)@3	-0,00434444
	ATKEQLKAVMDDFAAFVEK	Methacrylic anhydride(K)@7	-0,00434444
	ATKEQLKAVMDDFAAFVEK	Methacrylic anhydride@N-term; Methacrylic anhydride(K)@3	-0,000122901
	ATKEQLKAVMDDFAAFVEK	Methacrylic anhydride@N-term; Methacrylic anhydride(K)@3; Methacrylic anhydride(K)@7	-0,0088598
	ATKEQLKAVMDDFAAFVEK	Methacrylic anhydride(K)@3; Methacrylic anhydride(K)@7	0,00994787
	CCKADDKETCFAEEGKK	Methacrylic anhydride(K)@3	-0,026782401
	DAHKSEVAHR	Methacrylic anhydride(K)@4	-0,0268817
	DAHKSEVAHR	Methacrylic anhydride@N-term; Methacrylic anhydride(K)@4	-0,0338878
	DAHKSEVAHR	Methacrylic anhydride(H)@3; Methacrylic anhydride(K)@4	-0,0338878
	DDKETCFAEEGKK	Methacrylic anhydride(K)@3	0,0292862
	DDNPNLPR	Methacrylic anhydride@N-term	-0,0147698
	DVCKNYAEAK	Methacrylic anhydride(K)@4	-0,00347931
	EFNAETFTFHADICTLSEKER	Methacrylic anhydride(R)@21	0,026110001
	EFNAETFTFHADICTLSEKER	Methacrylic anhydride(K)@19	-0,0147899

	EQLKAVMDDFAAFVEK	Methacrylic anhydride(K)@4	0,00153408
	EQLKAVMDDFAAFVEK	Methacrylic anhydride(Q)@2	0,024809999
	FKAWAVAR	Methacrylic anhydride(K)@2	-0,0136292
	FKDLGEENFK	Methacrylic anhydride(K)@2	-0,00858875
	FKDLGEENFK	Methacrylic anhydride@N-term	0,0229425
	FPKAEFAEVSK	Methacrylic anhydride(K)@3	-0,0064321
	FPKAEFAEVSK	Methacrylic anhydride@N-term; Methacrylic anhydride(K)@3	0,00146627
	FQNALLVR	Methacrylic anhydride@N-term	-0,0133743
	FQNALLVR	Methacrylic anhydride(Q)@2	-0,0146548
	FYAPELLFFAKR	Methacrylic anhydride(K)@11	-0,0170558
	HPDYSVVLLLR	Methacrylic anhydride(H)@1	-0,0114803
	HPDYSVVLLLR	Methacrylic anhydride@N-term	-0,0114803
	HPYFYAPELLFFAK	Methacrylic anhydride(H)@1	-0,0117885
	HPYFYAPELLFFAK	Methacrylic anhydride@N-term	-0,0117885
	HPYFYAPELLFFAK	Methacrylic anhydride(H)@1	0,0227591
	HPYFYAPELLFFAKR	Methacrylic anhydride(R)@15	0,025612701
	KEQLKAVMDDFAAFVEK	Methacrylic anhydride(K)@1	0,000375254
	KEQLKAVMDDFAAFVEK	Methacrylic anhydride(Q)@3	-0,000252954
	KEQLKAVMDDFAAFVEK	Methacrylic anhydride(K)@5	0,00656782
	KQTALVELVK	Methacrylic anhydride(Q)@2	-0,0120111
	KQTALVELVK	Methacrylic anhydride(K)@1	-0,0126101
	KQTALVELVK	Methacrylic anhydride(K)@1; Methacrylic anhydride(Q)@2	-0,0358514
	KVPQVSTPTLVEVSR	Methacrylic anhydride(K)@1	-0,0146041
	KVPQVSTPTLVEVSR	Methacrylic anhydride(Q)@4	-0,0146041
	KVPQVSTPTLVEVSR	Methacrylic anhydride(K)@1; Methacrylic anhydride(Q)@4	-0,00249775
	KYLYEIAR	Methacrylic anhydride(K)@1	-9,45E-05
	LAKTYETTLEK	Methacrylic anhydride(K)@3	-0,0135266
	LAKTYETTLEKCCAAADPHECYAK	Methacrylic anhydride(K)@3; Methacrylic anhydride(K)@11	0,00145459
	LCTVATLR	Methacrylic anhydride@N-term	-0,0147281
	LDELRDEGK	Methacrylic anhydride@N-term	0,00400725
	LDELRDEGKASSAK	Methacrylic anhydride(K)@14	-0,0157097
	LDELRDEGKASSAK	Methacrylic anhydride(K)@9	-0,0157097
	LDELRDEGKASSAK	Methacrylic anhydride(R)@5	0,020675801
	LDELRDEGKASSAKQR	Methacrylic anhydride(K)@9; Methacrylic anhydride(K)@14	0,00351159
	LECADDRADLAKYICENQDSISSK	Methacrylic anhydride(K)@12	-0,0217715
	LVAASQAALGL	Methacrylic anhydride@N-term	-0,01296
	LVNEVTEFAK	Methacrylic anhydride@N-term	-0,0111327
	LVNEVTEFAKTCVADESAENCDK	Methacrylic anhydride(K)@10	-0,0105955
	LVRPEVDVMCTAFHDNEETFLKK	Methacrylic anhydride(K)@22	-0,023963399
	NECFLQHKDDNPNLPR	Methacrylic anhydride(Q)@6	0,0254297
	NECFLQHKDDNPNLPR	Methacrylic anhydride(H)@7	0,0254297
	NECFLQHKDDNPNLPR	Methacrylic anhydride(K)@8	0,00197763
	QIKKQTALVELVK	Methacrylic anhydride(Q)@5	-0,0066451
	QIKKQTALVELVK	Methacrylic anhydride(K)@3; Methacrylic anhydride(K)@4	-0,038372699
	QIKKQTALVELVK	Methacrylic anhydride(K)@4	0,023495

	QIKKQTALVELVK	Methacrylic anhydride(K)@3	0,023495
	QNCELFEQLGEYK	Methacrylic anhydride(Q)@1	-0,00738804
	QNCELFEQLGEYK	Methacrylic anhydride@N-term	-0,00738804
	QNCELFEQLGEYK	Methacrylic anhydride(N)@2	-0,0126843
	QNCELFEQLGEYKFQNALLVR	Methacrylic anhydride(K)@13	-0,00931356
	QNCELFEQLGEYKFQNALLVR	Methacrylic anhydride(Q)@15	0,0120424
	QNCELFEQLGEYKFQNALLVR	Methacrylic anhydride(K)@13; Methacrylic anhydride(Q)@15	-0,000807693
	QNCELFEQLGEYKFQNALLVR	Methacrylic anhydride(Q)@8	0,023444301
	RAFKAWAVAR	Methacrylic anhydride(K)@4	-0,0103486
	RHPDYSVVLLLR	Methacrylic anhydride(H)@2	-0,019065799
	RHPDYSVVLLLR	Methacrylic anhydride@N-term	-0,0134026
	RHPDYSVVLLLR	Methacrylic anhydride(R)@1	-0,0134026
	RHPDYSVVLLLR	Methacrylic anhydride(H)@2	-0,0165489
	RYKAAFTECCQAADK	Methacrylic anhydride(K)@3	-0,0102994
	SLHTLFGDKLCTVATLR	Methacrylic anhydride(K)@9	-0,0040148
	SLHTLFGDKLCTVATLR	Methacrylic anhydride(H)@3; Methacrylic anhydride(K)@9	0,0080417
	SLHTLFGDKLCTVATLR	Methacrylic anhydride@N-term; Methacrylic anhydride(H)@3	0,00659075
	SLHTLFGDKLCTVATLR	Methacrylic anhydride@N-term; Methacrylic anhydride(K)@9	-0,00152457
	SLHTLFGDKLCTVATLR	Methacrylic anhydride(H)@3	0,0125291
	SLHTLFGDKLCTVATLR	Methacrylic anhydride(H)@3	0,022907101
	TCVADESAENCDK	Methacrylic anhydride(K)@13	0,0128826
	TECCHGDLLECADDRADLAK	Methacrylic anhydride@N-term; Methacrylic anhydride(H)@5	0,010497
	TKCCTESLVNR	Methacrylic anhydride(K)@2	0,022470299
	TKEQLKAVMDDFAAFVEK	Methacrylic anhydride@N-term; Methacrylic anhydride(K)@2; Methacrylic anhydride(K)@6	-0,0146839
	TKEQLKAVMDDFAAFVEK	Methacrylic anhydride(K)@2; Methacrylic anhydride(Q)@4; Methacrylic anhydride(K)@6	-0,0146839
	TKEQLKAVMDDFAAFVEK	Methacrylic anhydride(K)@2	-0,00918596
	TKEQLKAVMDDFAAFVEK	Methacrylic anhydride(K)@2; Methacrylic anhydride(K)@6	0,00451451
	TKEQLKAVMDDFAAFVEK	Methacrylic anhydride(K)@2; Methacrylic anhydride(Q)@4	0,00147543
	TKEQLKAVMDDFAAFVEK	Methacrylic anhydride(Q)@4	0,00272648
	VFDEFKPLVEEPQNLIK	Methacrylic anhydride@N-term	-0,0116931
	VFDEFKPLVEEPQNLIK	Methacrylic anhydride(K)@6	-0,018092999
	VFDEFKPLVEEPQNLIKQNCELFEQLGEYK	Methacrylic anhydride(K)@17	0,020436101
	VGSKCCKHPEAK	Methacrylic anhydride(K)@4	-0,025557499
	VGSKCCKHPEAK	Methacrylic anhydride(K)@7	-0,0210864
	VNEVTEFAK	Methacrylic anhydride(N)@2	0,0136247
	VPQVSTPTLVEVSR	Methacrylic anhydride@N-term; Methacrylic anhydride(Q)@3	0,00652401
	VTKCCTESLVNR	Methacrylic anhydride(K)@3	0,00147493
	YICENQDSISSKLK	Methacrylic anhydride(K)@14	0,0205606
	YICENQDSISSKLK	Methacrylic anhydride(K)@12	0,0205606
	YKAAFTECCQAADK	Methacrylic anhydride@N-term	0,0197674
	YKAAFTECCQAADK	Methacrylic anhydride(K)@2	0,00811773
	YTKKVPQVSTPTLVEVSR	Methacrylic anhydride(K)@3	0,00508104
	YTKKVPQVSTPTLVEVSR	Methacrylic anhydride(K)@4	0,00508104
	YTKKVPQVSTPTLVEVSR	Methacrylic anhydride(K)@3; Methacrylic anhydride(K)@4	-0,00749501
Complement C3	EPGQDLVVLPLSITTDFIPSFR	Methacrylic anhydride(Q)@4	-0,0181063

	IFTVNHKLLPVGR	Methacrylic anhydride(K)@7	-0,00717962
	QGALELIKK	Methacrylic anhydride(K)@8	0,0243588
	QGALELIKK	Methacrylic anhydride(K)@9	0,0243588
Serotransferrin	CLKDGAGDVAFVK	Methacrylic anhydride(K)@3	-0,0222151
	KSASDLTWDNLK	Methacrylic anhydride(K)@1	-0,0129697
	NLNEKDYELLCLDGTR	Methacrylic anhydride(K)@5	0,00147754
	NPDPWAKNLNEKDYELLCLDGTR	Methacrylic anhydride(K)@12	-0,0145057
	SVIPSDGPSVACVKK	Methacrylic anhydride(K)@15	-0,0109093
	KSASDLTWDNLKGK	Methacrylic anhydride(K)@12	0,0128116
	CLKDGAGDVAFVK	Methacrylic anhydride(K)@3	-0,0035064
	GWNIPIGLLYCDLPEPR	Methacrylic anhydride(W)@2; Methacrylic anhydride(N)@3	-0,0236942
	KASYLDCIR	Methacrylic anhydride(K)@1	-0,0077725
	KCSTSSLLEACTFR	Methacrylic anhydride(K)@1	-0,0110026
	KDSGFQMNQLR	Methacrylic anhydride(K)@1	0,00363786
	MDAKMYLGYEYVTAIR	Methacrylic anhydride(K)@4	0,00383966
	NPDPWAKNLNEKDYELLCLDGTR	Methacrylic anhydride(K)@7	-0,0145057
	SASDLTWDNLKGK	Methacrylic anhydride(K)@11	-0,023888201
	SKEFQLFSSPHGK	Methacrylic anhydride(K)@2	0,0088012
	SVIPSDGPSVACVKK	Methacrylic anhydride(K)@14	-0,0215207
Alpha-2-macroglobulin	GHFSISIPVKSDIAPVAR	Methacrylic anhydride(K)@10	-0,017948
	GVPIPNKVIFIR	Methacrylic anhydride(K)@7	-0,00469111
Alpha-1-antitrypsin	LSITGTYDLKSVLGQLGITK	Methacrylic anhydride(K)@10	0,00175891
	GTQGKIVDLVK	Methacrylic anhydride(K)@5	-0,0071879
	LVDKFLEDVKK	Methacrylic anhydride(K)@4	0,00242497
	LVDKFLEDVKK	Methacrylic anhydride(K)@10	-0,0221914
	SVLGQLGITKVFSNGADLSGVTEEAPLK	Methacrylic anhydride(K)@10	-0,0122591
Haptoglobin	FTDHLKYVMLPVADQDQCIR	Methacrylic anhydride(K)@6	-0,00149392
	HYEGSTVPEKK	Methacrylic anhydride(K)@10	-0,023800701
	DIAPTLTLYVGKK	Methacrylic anhydride(K)@12	-0,0261031
	DIAPTLTLYVGKK	Methacrylic anhydride(K)@13	0,0233745
	HYEGSTVPEKK	Methacrylic anhydride(K)@11	0,0238182
	HYEGSTVPEKK	Methacrylic anhydride(K)@10	-0,021717001
	ILGGHLDAKGSFPWQAK	Methacrylic anhydride(K)@9	-0,010681
Apolipoprotein A-I	VKDLATVYVDVLK	Methacrylic anhydride(K)@2	-0,00621585
	LAARLEALKENGGAR	Methacrylic anhydride(K)@9	-0,00251401
	AKPALEDLR	Methacrylic anhydride@N-term	0,026486499
	AKVQPYLDDFQKK	Methacrylic anhydride@N-term	0,023417801
	KWQEEMELYR	Methacrylic anhydride(W)@2	0,025477899
	KWQEEMELYR	Methacrylic anhydride(K)@1	8,98E-05
	LEALKENGGAR	Methacrylic anhydride(K)@5	-0,026768699
	LEALKENGGAR	Methacrylic anhydride(R)@11	-0,026768699
	VKDLATVYVDVLK	Methacrylic anhydride(K)@2	-0,00422069
Complement C4-B	DHAVDLIQKGYMR	Methacrylic anhydride(K)@9	-0,00509481

Table 2. MA modified peptides and correlated proteins for PLMA300 modification.