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Blood plasma derivatives for tissue engineering and regenerative medicine therapies

SC Santos¹, Ólafur Eysteinn Sigurjonsson², Catarina Almeida Custódio^{1,*}, and João Filipe Mano^{1,*}

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

²1) The Blood Bank, Landspítali University Hospital, Snorrabraut 60, 101 Reykjavik, Iceland 2) School of Science and Engineering, University of Reykjavik, Menntavegur 1, 101 Reykjavik

Abstract

Platelet-rich plasma (PRP) and its derivatives have been 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 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 growth factors. In the last years, it has been witnessed a great development on PRP based biomaterials, with both structural and functional purposes. Here 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 fields of tissue engineering and regenerative medicine and provides a perspective on future directions.

1 Platelets

Platelets are key blood elements and renowned for their central role in haemostasis. They are a natural reservoir of multiple proteins, cytokines and growth factors (GFs) that are stored in a dense membrane system e.g. stored in the α -granules within them. *In vivo*, platelets are activated at the site of injury where they participate in haemostasis, promoting tissue repair, including revascularization^{1,2}. GFs extraction through lysis of human platelets represents a less expensive and safer alternative to the use of recombinant GFs or animal proteins and GFs³. These signaling molecules, when in contact with cell transmembrane receptors promote expression of genes involved in processes like cellular recruitment, growth and morphogenesis⁴. Other molecules present in platelet α -granules include adhesive proteins, clotting factors, fibrinolytic factors, proteases and antiproteases and membrane glycoproteins⁵. CD40 ligand and p-selectin molecules involved in inflammation processes are also a component of the platelet α -granules that are released upon platelet activation^{6,7}.

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

Overall, platelet activation allows the release of a large amount of soluble mediators, that are essential in wound healing, stimulates clotting, and angiogenesis. Therefore, from a therapeutic point of view, the use of platelets seems to be quite promising for regenerative medicine and tissue engineering applications.

For therapeutic applications, platelets are commonly used in the form of platelet rich plasma (PRP). In PRP the normal concentration of platelets is at least 1×10^6 platelets/ μL in 5 mL of plasma, while in whole blood range from 1.5×10^5 platelets/ μL to 3.5×10^5 platelets/ μL ^{8,9}. First reports on the use of PRP as a possible therapy are from 1990s, with its application in cardiac surgery¹⁰ and also in bone grafts¹¹. Nowadays, there are several areas where the potential therapeutic effect of PRP is documented: dentistry¹², orthopedics¹³, neurology¹⁴, plastic surgery¹⁵, musculoskeletal conditions^{13,16} and also *in vitro* fertilization¹⁷.

2 Preparation of PRP and platelet lysates

PRP is easily obtained by a differential centrifugation of a whole blood sample^{9,18,19}. Platelet activation is then imperative for the release of the soluble factors. Platelet activation is usually achieved by three different methods, namely the addition of calcium chloride and thrombin, contact with collagen or freeze/thaw cycles (Figure 1).⁹ Compared to chemical activation of platelets which may cause side effects, mechanical lysis via freezing and thawing it is easier, less time-consuming and also cost-effective²⁰.

3 Biochemical composition of platelet rich plasma and platelet lysate

The major component of plasma is albumin and globulins that act as carriers for various biomolecules, other components include fibrinogen that plays an important role in blood clotting cascade^{8,9}. GFs present in human PRP and PL include platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF)²¹, insulin-like growth factor I and II (IGFI and II), TGF- β ²², epidermal growth factor (EGF), epithelial cell growth factor (ECGF)²³, connective tissue growth factor (CTGF), platelet factor 4 (PF4), interleukin 8 (IL-8) and keratinocytes growth factor (KGF)²⁴. These GFs perform function such as chemoattraction, cell proliferation and maturation, matrix synthesis and angiogenesis^{9,25}. Also clotting factors are present – factor V, factor XI, protein S and antithrombin – and all of them are responsible for thrombin activation and fibrin clot formation⁸.

4 Platelet lysate 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^{31–33}

Cells from bone marrow³⁴, adipose tissue^{34,35}, trabecular bone³⁴, dental pulp³⁴, mesenchymal stem cells (MSCs)^{36–39}, human articular chondrocytes³⁵, myofibroblasts⁴⁰, human immortal keratinocyte cell line⁴¹ and osteoblasts⁴² respond to the use of PL with increased proliferation rate when compared to animal-derived mediums or mixtures of recombinant GFs^{31–34,43}. It was also reported that the use of PL in substitution of FBS do not interfere with the differentiation potential and immunophenotypic characteristics, at least in case of MSCs⁴³.

The proliferation process is highly 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. PL contain a total protein content typically exceeding 50–55 mg/mL while FBS composition includes a protein content that is on average 38 mg/mL, this may explain the higher proliferation rates in cells cultured using PL as a culture media supplement.

Despite the promising results, a major concern regarding the use of PL is their heterogeneity and demanding characterization. PL quality control procedures are urgently needed, which could include measurements of key molecules important for cell growth and differentiation. Advanced characterization techniques such as mass spectrometry and two-dimensional gel electrophoresis and enzyme-linked immunosorbent analyses could be important for a better characterization and standardization in the use of PL^{18,29}. Testing for infectious markers or pathogen inactivation are also important processes in PL preparation. In case of infectious markers, each PL sample should be submitted to the detection analysis of human immunodeficiency virus (HIV) antibodies, hepatitis B surface antigen, hepatitis B virus and hepatitis C virus antibodies. Pathogen inactivation during PL obtaining process reduces risk of pathogens transmission such as viruses, bacteria, fungi and prions.

Frequently, human PL used as a supplement for cell culture media is obtained from processed blood donations from FDA or EMA licensed blood centers. Is also now possible to obtain commercial sources of human PL to replace FBS, e.g. PLTMax® from Merck, Human Platelet Lysate from Stem Cell Technologies™ and CellAdvance® hPL from BBISolutions. In all the cases, preferably multiple donor units are pooled during manufacturing to minimize lot-to-lot variability.

5 PRP and fibrin based materials

When activated *in vivo*, platelets release a rich cocktail of proteins initializing the coagulation cascade that leads to formation of a gel-like network of fibrin (Figure 2). *In vivo*, fibrin gels act as a scaffold, providing tissue repair and important cues for directing cell phenotype following injury. This matrix can be broken down by plasmin enzyme through a process called fibrinolysis^{9,44}. 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 sealants⁴⁵. 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 attachment⁴⁶.

Fibrin gel network forms aligned fibrils in direction of extension and perpendicular to the direction of compression, with mechanical properties particularly appealing for engineering vascular tissue and cardiac patches^{47–49}. Tranquillo *et al* have used fibroblasts cultured in fibrin hydrogels to engineer vascular grafts. In this system, the fibrin gels were cultured in a pulsed flow-stretch bioreactor, to induce circumferential tissue alignment that correlate with graft strengthening⁵⁰. More recently, Atala *et al* reported the use of 3D bioprinting to prepare fibrin patches to engineer cardiac tissue. Their results show that the bioprinted patches were able to contract and beat like a normal heart after 4 weeks in culture⁵¹.

Hybrid hydrogels of fibrin and hyaluronic acid were proposed by T. N. Snyder *et al* as a suitable three-dimensional environment for the proliferation of bone marrow-derived MSCs⁵². For skeletal muscle regeneration, Marcinczyk *et al* proposed fibrin based gels enriched with laminin to promote myoblast proliferation and alignment ⁵³.

As previously referred in this review, PL based gels may be formed by the addition of calcium and thrombin that triggers a coagulation cascade, promoting fibrinogen polymerization in fibrin clots. A pioneer work from G. Walenda *et al* reported about PL based gel as a soft matrix for mesenchymal stromal cells expansion²². Since then other works have also demonstrated that PL gels support the formation of fully human capillary network, establishing a *de novo* endothelial network and lumen present in capillaries, and led to a rapid neovascularization of ischemic tissues^{3,54}. There may be explained by the presence of a series of pro-angiogenic molecules such as VEGF, PDGF and SDF-1 on PL. Overall, these findings suggest that the relatively soft and viscoelastic nature of PL gel combined with the growth factor milieu, hold promise in promoting stem cell expansion and invasion and enhance vascular regeneration. Furthermore, mechanical analysis have shown that PL gels can have a very soft and viscous consistency with increasing elasticity with the increase of PL percentage²². Despite all the great intrinsic properties of PL based gels, this type of material is generally associated with weak mechanical properties and poor stability *in vivo* and cell culture conditions⁵⁵. 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^{23,56}. 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 the PL based biomaterials up to the date. 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.

6 Hybrid PRP 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 release of GFs. This may represent important biological and economic advantages, as recombinant GFs widely used are very expensive and may promote immunogenic reactions 57,58. Methods to create three-dimensional matrices with incorporated PL include incubation of the polymeric matrices with PL solutions or mixing PL with the polymeric precursor before curing¹⁸. 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 cross-links between the polymer chains. Chemical cross-linking 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^{59–61}. 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 PRP⁶². 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.

R. Ito *et al* proposed a collagen/gelatin scaffold impregnated with PL to accelerate the formation of dermis-like tissue in mice⁶³. Their findings indicate that collagen/gelatin scaffold bind to TGF- β and PDGF-BB from the PL 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 three-dimensional constructs²¹. Interestingly, they also report the use of microfluidic tools to design gradients of PL along gellan gum hydrogel fibers. Results from the culture of endothelial cells within the fibers, indicate that these cells respond to the gradient of PL, tending to adhere and growth in regions with higher content of growth factors. A hydrogel based on PL loaded chitosan-chondroitin sulfate nanoparticles was also reported by the same group⁶⁴. Biological assays showed that the hydrogels maintained cell viability and that PL can work as a GFs supplement for cell proliferation. The use of human PLs to build these aggregates can also provide mechanical stability to the three-dimensional 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.

J. Leotot *et al* reported how PL can influence MSCs behavior if used as a coating of hydroxyapatite/ β -tricalcium phosphate scaffolds⁶⁵. Not only a higher number of MSCs adhered to the scaffold but also cells had increased cytoplasmic extensions after few hours in culture. Proliferation and osteogenic differentiation of stem cells was also increased by the presence of PL. Their findings provide evidence of the potential of PL to develop novel clinical strategies for bone repair²⁸.

In another attempt to combine PL with biomaterials towards bone regeneration, S. Oliveira *et al* have prepared polycaprolactone (PCL) scaffolds that were modified with PL and

marine-origin polysaccharides using layer-by-layer (LbL) 58. Their results demonstrate that the modified 3D scaffolds induced adipose derived stem cells differentiation into mature osteoblasts. They also observed that the fibrillar structures with PL allowed a three-dimensional organization of the mineralization. The same authors reported the buildup of PL nanocoatings prepared by LbL assembling on different marine derived polysaccharides. This type of hierarchical scaffolds demonstrated also pro-angiogenic properties, promoting formation of tubular structures in endothelial cells⁶⁶.

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 cells⁶⁷. 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 GFs⁶⁸. The functional particles assembled into a stable three-dimensional construct triggered by the presence of adipose derived stem cells, 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 PRP

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 sternotomy⁶⁹. 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 tears⁷⁰, knee osteoarthritis^{13,71}, ulnar collateral ligament tears⁷², lateral epicondylitis⁷³, hamstring injuries⁷⁴ and Achilles tendinopathy⁷⁵. Different methods have been used in order to alleviate symptoms of knee osteoarthritis patients, including the use of PRP. Raeissadat *et al* carried out a randomized clinical trial and results showed the efficacy of PRP injections in reducing symptoms in patients with knee osteoarthritis⁷¹. Also Huang *et al*, in a similar study, demonstrated the beneficial effects of PRP in repairing joint tissue and reduction of joint inflammation, cartilage destruction and also in bone damage¹³. 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 procedure⁷⁶. 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*⁷⁷ 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 elbow⁷⁸. 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 stromal 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 nonunion⁷⁹. 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^{80,81}. 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 in the use of autologous PRP in chronic nonhealing leg ulcers treatment⁸². 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 faster⁸³.

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. Gentile⁸⁴ studied the influence of PRP in this pathology. The treatment with PRP mixed with fat tissue favors tissue growth and also three-dimensional 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 recovery⁸⁵. S. Anjayani *et al* carried out a study on the effectiveness of PRP injections in patients with leprosy peripheral neuropathy⁸⁶. 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.

Considering all the promising results on the use of PRP/PL in clinical practices and in tissue engineering research area some companies have been not only developing new systems for isolation of PRP/PL but also developing new strategies for the use of such blood derived products. REgenLab®USA, PRP & Cell Therapy Specialist developed the RegenKit® BCT which allows the isolation of PRP from a small whole blood sample of the patient in an easy way. The obtain PRP is then mixed with autograft and / or allograft bone and then applied to a bony defect. In another approach, Platome – World of Platelets provides solution for cell culture free of animal serums. The use of human derived products instead of animal derived

allows to an easy translation to clinical practices by the use of an environment that mimics what happens *in vivo*.

Despite all of the efforts trying to emphasize the use of blood derived products, there is still a need to develop new strategies and new approaches for the use of these sources. More research in tissue engineering and regenerative medicine areas, for example, is a way to develop new biomaterials and new ways to use such kind of blood derived products.

8 Conclusions and future directions

Tissue regeneration is a complex and coordinated physiological process involving a number of molecular, cellular and biochemical mechanisms. A variety of molecules are involved in tissue regeneration and wound healing, such as FGF, IGF-1, TGF- β 1, VEGF and BMPs, these growth factors are known for their adhesion, proliferation and differentiation effects on various cells. PL provide a combination of many of these factors, being a key player in tissue regeneration and wound healing strategies.

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.

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Figure 1. Schematic representation of PRP isolation and PL preparation. Platelet activation process. Substances like thrombin, calcium chloride and collagen or freeze-thaw cycles can activate or/and lyse platelets. Activated/lysed platelets release some molecules like cytokines or growth factors that are involved on cellular recruitment, growth and morphogenesis.

Figure 2.

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).

Figure 3.
PRP/PL acquisition process and PRP/PL based biomaterials and clinical applications.

Table 1

GFs, cytokines and chemokines present in PRP and GFs, cytokines/chemokines, adhesive proteins and clotting factors present in human platelet α -granules^{1,24–30}.

	Molecule Category	Molecule	Biologic Activity
PRP	Growth Factors	TGF- β 1, 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- β 1 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-1 α , GRO- α , ENA-78, MCP-1, 2 and 3, IL-1 β , CXCL6, SDF-1 α , PF4, neutrophil 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, XI, XIII, protein S and antithrombin	Thrombin activation and fibrin clot formation
	Immunoglobulins (Ig)	IgG, IgA, IgM	Immune mediators
	Other proteins	Albumin	Carrier protein