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Research Paper

Platelet lysate formulations based on mucoadhesive polymers for the treatment of corneal lesions

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Abstract

Objectives Growth factors contained in platelet α-granules initiate and modulate tissue repair and are proposed for the treatment of soft and hard-tissue surgical conditions and in the management of non-healing wounds. Platelet lysate is a hemoderivative obtained from platelet-rich plasma and is capable of releasing a pool of growth factors. Many medical and surgical techniques have been proposed for the treatment of corneal lesions; management of these conditions remains problematic and healing with standard protocols is unattainable. The aim of this study was to develop formulations suitable for prolonging the contact of platelet lysate with the damaged cornea for the time necessary to exert a therapeutic effect.
Methods Two vehicles, one based on polyacrylic acid and one based on chitosan, were autoclaved and loaded with platelet lysate and the resultant formulations were characterized for rheology, mucoadhesion, vehicle compatibility and stability. The proliferation effect was tested on two cell culture types (rabbit corneal epithelial cells and fibroblasts). An in-vitro wound-healing test was performed on fibroblasts. In both cases the formulations were compared with platelet lysate diluted with saline at the same concentration.
Findings Both formulations maintained the rheological and mucoadhesive properties of the vehicles and the proliferative activity of platelet lysate. The chitosan formulation was able to significantly enhance epithelial cell growth even after storage of up to 2 weeks (in-use conditions), while the polyacrylic acid formulation was less efficient, probably due to the characteristics of the cell model used.
Conclusions The in-vitro wound-healing test performed on fibroblasts confirmed the differences between the two vehicles. The effect induced by the platelet lysate and chitosan formulation was faster than that of the polyacrylic acid formulation and complete in-vitro wound repair was achieved within 48 h.
Keywords chitosan; cornea; growth factors; polyacrylic acid; wound healing

Introduction

Platelets are specialized secretory cells that release, in response to activation, a large number of biologically active substances from intracellular alpha granules. Among these substances is the very important category of growth factors (GFs). These initiate and modulate tissue repair mechanisms, such as chemotaxis, cell proliferation, angiogenesis, extracellular matrix depositing and remodelling.[1,2] A large number of platelet GFs have, until now, been isolated, studied and characterized. Among these, the most intensively investigated are platelet derived growth factor (PDGF), transforming growth factors alpha and beta (TGF-α and -β), platelet-derived epidermal growth factor (PDEGF), epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF). Some of these GFs are available in purified form, but it has been observed that tissue repair cannot be mediated effectively by a single agent, as multiple signals are required to complete the regeneration process.[1] To better exploit the whole potential of the naturally occurring platelet GFs, the therapeutic employment of platelet-rich preparations has been suggested. These are hemoderivatives, from which platelets can release their complete pool of biologically active substances.

Among these hemoderivatives is platelet-rich plasma (PRP), which consists of a limited volume of plasma enriched in platelets, which can be obtained from the patient (autologous)

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or from donors (allogenic). The platelet concentrate can therefore be activated by adding thrombin or calcium to form a three-dimensional and biocompatible fibrin scaffold (fibrin glue). The term 'releasate' is usually used to describe the preparations, which consist of bioactive molecules in a solution obtained by activation of platelets with calcium or thrombin, while the term 'lysate' indicates the solution of bioactive molecules obtained by platelet destruction by freeze-thawing, usually starting from a PRP sample in the presence of an anticoagulant agent.[2–4]

Despite some controversial results,[5,6] partially due to the need for a better standardization of the products,[7] many reports describe the efficacy of platelet-rich preparations in skin wound healing, in the repair of orthopaedic and periodontal lesions, and in ocular treatment.[8–12] It is recognized that the efficacy of the GFs critically depends on the way they are made available to the injured tissue. The development of suitable therapeutic vehicles is therefore of paramount importance to the release of GFs according to the repair requirements.[1]

Single or combined isolated GFs have been delivered in different release systems,[13–16] but very few references can be found in the literature about the combination of hemoderivatives and, in particular, of platelet lysate (PL) with mucoadhesive materials. The combination of PL and mucoadhesive polymers can be useful for the application to mucosal surfaces, such as those involved in the oral mucosities that often occur during intensive chemotherapy or as a consequence of the graft versus host disease (GvHD) that follows transplantation,[17,18] and to improve the residence time in the corneal area.

Corneal alteration can occur as a consequence of neurodystrophic keratitis, surgical intervention, or as a chronic symptom of GvHD. Persistent corneal epithelial defects (CEDs) are associated with decreased production of tears and reduced corneal sensitivity. CEDs cause significant pain and visual impairment, are often unresponsive to conventional treatments and are therefore difficult for ophthalmologists to treat.[19]

In the cornea, after the epithelial injury, the wound healing cascade involves stromal-epithelial and immune cell interactions, mediated by cytokines, GFs, chemokines and keratocyte apoptosis. Tear film also plays an important role. The injured epithelium and epithelial basement membrane releases IL-1 and TNF-α, bone morphogenic proteins (BMP) 2 and 4, EGF and PDGF, which induce keratocyte apoptosis in the underlying stroma. After the initial wave of keratocyte apoptosis, increasing numbers of cells undergo the more proinflammatory process of necrosis. Proliferation and migration of the remaining keratocytes begins within 12–24 h, giving rise to activated keratocytes, fibroblasts and possibly myofibroblasts, responsible for repopulating the depleted stroma. As wound healing switches from the inflammatory phase to the proliferative phase, corneal fibroblasts accomplish the transition from a proinflammatory state to a profibrotic state.[20] Also within the first 24 h of injury, there is a stromal infiltration by macrophages/monocytes, T cells and polymorphonuclear cells, which play a role in phagocytosis of apoptotic and necrotic debris. One to two weeks following injury, myofibroblasts, which derive from keratocytes responding to TGF-β, appear and remodel collagen and extracellular matrix through production of collagen, glycosaminoglycans, collagenases, gelatinases and matrix metalloproteinases (MMP). The myofibroblasts slowly disappear over the ensuing weeks, although the process may continue for months to years.[21]

Abnormal regulation of healing can lead to serious complications, such as keratectasia or loss of corneal transparency (severe haze).[21]

Although many medical and surgical techniques have been proposed for the treatment of corneal lesions, the management of these conditions remains problematic and healing with standard protocols is unattainable.[22] Growth factors stored in platelet α-granules at high concentrations are able to manifest all their regenerative and repairing potential, triggering a healing process that starts and amplifies the lesion resolution, stimulating effects such as angiogenesis, chemotaxis of macrophages, proliferation and migration of fibroblasts and the synthesis of collagen.[22]

The aim of the present work was therefore to develop formulations suitable to maintain PL, rich in GFs and other bioactive molecules involved in the healing process, in contact with injured tissues for a time sufficient to treat the lesions. This represents a challenge, especially when the application site is the cornea. In this case the residence time is crucial to allow active molecules to maintain and prolong the contact with the injured tissues for a sufficient time to exert the therapeutic effect. For these reasons a combination of PL with biomaterials, which is able to fulfil the above prerequisite is mandatory. For these purposes, polyacrylic acid (PAA) and chitosan were selected as base materials because of their well-known mucoadhesive properties. The wound-healing properties of chitosan have also already been documented.[23,24]

A prerequisite of formulations intended for ophthalmic administration is sterility. In this study, rheological and mucoadhesive properties were featured to improve resistance towards the removal effects of lachrymation and blinking. The compatibility and the eventual synergic effect of the PL with the vehicles was evaluated by comparing, on appropriate cell cultures of rabbit corneal epithelial (RCE) cell line and fibroblast line, the proliferative effect of PL alone and incorporated in the vehicle. This evaluation was performed soon after the preparation and during a storage period of 2 weeks at 2–8°C (storage temperature) to simulate in-use conditions. Platelet lysate is in fact normally incorporated in the vehicle at the hospital when the patient comes for treatment of the lesions. To ensure sterility, aliquots sufficient for each application are furnished to patients in sterile ophthalmic gel bottles that the patient can then brings home to use autonomously. Good stability (compatibility of GFs and vehicles and maintenance of proliferative and wound-healing capability) of the preparations means that the patient need only come back to the hospital for further treatment after the end of 2 weeks of self-treatment at home, with better patient compliance and lower health system costs resulting.

To more easily demonstrate the occurrence of an eventual modification of GF structure in the formulations and to assess if this could influence the biological effect and to what extent, a test based on the induction of proliferation on cell cultures was performed. RCE cell lines were chosen for this purpose.

The capability of formulations to enhance tissue regeneration was finally assessed by means of an in-vitro wound-healing test performed on a fibroblast cell line. Fibroblasts were chosen because, as a model of corneal fibroblasts, they exhibit different phenotypes in different phases of corneal wound healing. In the inflammatory phase, the cells assume a proinflammatory phenotype and produce large amounts of cytokines and chemokines, but in the proliferative and remodelling phases they adapt a profibrotic state, differentiate into myofibroblasts and increase extracellular matrix protein synthesis, secretion and deposition.[20] For the in-vitro wound-healing tests, cells were grown in a plate containing an insert designed to leave a gap of predetermined width inside the cell layer. The ability of the cells to grow inside this gap is a measure of the proliferative capacity of the culture in the presence of the test samples.

Materials and Methods

Materials

Polymers

Polyacrylic acid (PAA), Carbopol 974-PNF Batch # CC61NAB896, was obtained from Lubrizol (Brussels, Belgium). Chitosan glutamate 213 (CSG), (MW: 300 kDa, acetylation degree: 15%, acid glutammic content: 35–50%), was obtained from Protasan G213, Pronova Biomedical AS (Oslo, Norway). Hydroxypropylmethyl cellulose (HPMC): Methocel K100M CR Premium, was obtained from Colorcon Limited (Gallarate, Italy).

PL was obtained by the Apheresis Service of Immunohaematology and Transfusion Service Center for transplant immunology (Fondazione IRCCS Policlinico S. Matteo, Pavia) employing a sterile connection technique. Aliquots of hyperconcentrate platelets (high platelet concentration in small plasma volume and minimal leukocyte contamination) were obtained from apheresis performed on regular blood donors. The platelet pool was frozen at –80°C for 5 h and subsequently defrosted in a sterile water bath at 37°C, then diluted 1 : 1 with saline solution to obtain a final platelet concentration of approximately 500–600 $\times 10^3/\mu$l. An automated platelet count and tests for contaminations of aerobic and anaerobic bacteria and fungi, after saline dilution, were performed.

Preparation of vehicles and formulations

The PAA vehicle was prepared by dispersing PAA 5% w/w in saline solution (0.9% w/v NaCl). After complete dispersion of the PAA, the vehicle was buffered at pH 7.0 using NaOH (Ph. Eur. Grade; Riedel de Haen, Milano, Italy) solution 4 N.

The CSG vehicle was prepared by hydrating CSG at 6% w/w and HPMC at 2% w/w in saline solution (0.9% w/v NaCl). The pH of the CSG vehicle was not adjusted and was 5.5.

Both vehicles were sterilized using steam sterilization at 121°C for 15 min (Alpha Junior, PBI International, Milan, Italy). The formulations were prepared by mixing 1 : 1 (w/w) each vehicle with PL. The PL–PAA formulation was therefore obtained by mixing PL with PAA to obtain a final concentration of PAA equal to 2.5% w/w. The PL–CSG formulation was obtained by mixing the CSG and HPMC-based vehicle with PL and contained final concentrations of 3% w/w for CSG and 1% w/w for HPMC, respectively. In both cases the PL concentration was half of that present in the apheresis samples. The formulations were extemporaneous and were divided into aliquots in ophthalmic sterile gel bottles, which were useful for following the treatment schedule (one aliquot for each application for 15 days of treatment) to simulate in-use conditions. The aliquots were stored for 2 weeks at 2–8°C to simulate in-use conditions.

Assay of growth factors

The concentration of PDGF AB in the PL and in the formulations was assayed by means of the sandwich enzyme immunoassay technique (ELISA) test (Human PDGF AB Quantikine PharmPak, R&D systems, Minneapolis, MN; assay range: 31.2–2000 pg/ml). The concentration of PDGF AB in the formulations was related to the concentration of PDGF AB in the PL employed for their preparation and a parameter '% PDGF AB' was calculated as (concentration of PDGF AB in the formulation/concentration PDGF AB in PL) $\times$ 100.

Rheological characterization

The rheology of the vehicles and formulations was characterized by means of a rotational rheometer (Rheostress 600, Haake, Spinea, I). A cone plate combination (C35/1°) was used as a measuring system. All measurements were carried out at 25°C, after a rest time of 3 min. The apparent viscosity was measured by increasing shear rate values in the range 10–300 s^{-1}. Viscosity was measured on the vehicle after steam sterilization at 121°C for 15 min and on PL-loaded vehicles.

The pseudoplastic behaviour was evaluated and quantified, calculating the percentage decrease in viscosity at shear rates of 25 and 50 s^{-1} as follows:

$$(\eta_{25_{s-1}} - \eta_{50_{s-1}})/\eta_{25_{s-1}} \times 100$$

where $\eta_{25\,s-1}$ is the viscosity value at 25 s^{-1} and $\eta_{50\,s-1}$ is the viscosity at 50 s^{-1}.

Mucoadhesion measurements

Mucoadhesion measurements were performed using TA.XT plus (Texture analyser, ENCO, Spinea, Italy) equipped with a load cell of 1 kg and a cylinder probe of 1 cm and an A/MUC measuring system (mucoadhesion test ring). The A/MUC measuring system consists of a ring in which the biological support can be fixed. In this case the support was a filter paper disc wetted with 100 μl of mucin dispersion 8% w/w in phosphate buffer pH 6.4 (mucin type: type II crude, Sigma Aldrich, Milano, Italy). 20 mg of each sample was applied to the cylinder probe. Sample and biologic substrate were put in contact with a preload of 6000 mN for 3 min. The cylinder probe was moved upwards at a predetermined speed of 2.5 mm/min up to the complete separation of the mucoadhesive interface (mucin–sample).

The force of detachment as a function of displacement was recorded and the work of adhesion parameter (mN.mm) was calculated as the area under the force versus displacement

curve (AUC). The normalized parameter (ΔAUC/AUC) was calculated as follows:

$$\Delta AUC/AUC = (AUC_{mucin} - AUC_{blank})/AUC_{blank}$$

where AUC_{mucin} = AUC calculated in the experiment effected with biological substrate and AUC_{blank} = AUC calculated in the experiment effected without biological substrate. Such a parameter allows comparison of samples having different viscosity and therefore different cohesive properties; in particular, the normalization eliminates the contribution of viscosity to the strength of the mucoadhesive joint.[25]

Proliferation test

Rabbit corneal epithelial cell line (RCE) was obtained from the European Cell Culture Collection (N°95081046, ECACC, Salisbury, UK). Cells with passage numbers 8–12 were used. RCE cells were grown in a medium having the following composition: Dulbecco's Modified Eagle Medium (DMEM) mixed 1 : 1 with Ham's nutrient mixture F12, supplemented with L-glutamine (1% v/v, 2 mM), a mixture of penicillin (100 IU/ml), streptomycin (0.1 mg/ml) and amphothericin B (0.25 μg/ml), foetal bovine serum (15% v/v), EGF (10 ng/ml) and insulin (5 μg/ml) (Sigma, Milan, Italy). The cells were incubated at 37 ± 0.5°C in a humidified atmosphere containing 5% CO_2.

20 μl of RCE cell line suspension was seeded into each well of 96-well plates (area of 0.34 cm^2) at a density of about 7500 cells/well. A volume of 200 μl of each sample was simultaneously put into the well. The cells were co-seeded in the following media: complete growth medium (standard growth conditions), minimal medium (Mm, not supplemented with foetal calf serum) for the control; Mm containing PL diluted 1/20 and 1/40 (5% w/w and 2.5% w/w final concentration); Mm containing the formulation diluted to a PL concentration of 1/20 and 1/40 (5% w/w and 2.5% w/w).

The 96-well plate was kept at 37°C in an atmosphere of 95% air and 5% CO_2 and 95% relative humidity for 24 h. After 24 h, the cells were subconfluent and attached to the well bottom and a neutral red (NR) test was performed.

The NR assay (Tox Kit 4, Sigma-Aldrich, Milano I) determines the accumulation of NR supravital dye in the lysosomes of viable, uninjured cells. Cell membrane or lysosome damage causes poor or no capability to pick up NR.

Each well was washed with saline phosphate buffer (PBS) to remove surnatants. A volume of 200 μl of NR solution (0.33 mg/ml in DMEM) was put in each well for a contact time of 2 h. Cell substrates were then washed with PBS to eliminate NR not entrapped into cells, and the fixing medium (1% $CaCl_2$ and 0.5% formaldehyde aqueous solution) was added to fix the cell substrate. The fixing solution was then removed and a solubilizing solution (1% of acetic acid in ethanol) was added to each cell substrate to cause cell disruption and to release NR captured by viable cells. The NR solution absorbance was determined by means of an ELISA plate reader (Perkin Elmer, Milan, I) at a wavelength of 490 nm with a 650 nm reference wavelength. The absorbance read for each sample was compared with that of complete growth medium as the positive control (growth in standard conditions), which was considered to have 100% viability.[26,27] A comparison of the results obtained with PL–CSG and with PL–PAA on RCE and with PL–PAA on fibroblasts was performed by using as a parameter the ratio between the proliferation induced by the formulations and that induced by the PL apheresis sample employed to prepare the same formulation.

In-vitro wound-healing test

Normal human dermal fibroblasts from juvenile foreskin (PromoCell GmbH, Heidelberg, Germany) were used. Cells between the second and fifth passages were used for all the experiments. DMEM (Sigma, Milan, Italy) supplemented with 10% foetal calf serum (FCS, Sigma), 200 U/ml penicillin, and with 0.2 mg/ml streptomycin was used as the growth medium. The cells were kept at 37°C in a 5% CO_2 atmosphere with 95% relative humidity.

The in-vitro wound-healing test is based on the use of a Petri μ-dish (Ibidi, Giardini, Milan, Italy) in which an insert is enclosed. The insert is formed of two chambers with a growth area of 0.22 cm^2 divided by a septum with a width of cell-free gap of 500 μm ± 50 μm.

Fibroblasts were seeded in each chamber at 10^5 cells/cm^2 and growth at confluence in standard conditions as above mentioned. After 24 h, cells reach confluence and the insert is removed, displaying two areas of cell substrate divided by the prefixed gap. Cell substrates were put in contact with 200 μl of the formulation, diluted 1/20 with PL at 5 concentration, and the complete medium. At prefixed times (0, 24, 48, 72 and 98 h) microphotographs were taken to evaluate the invasion and cell growth in the gap.

The healing process was also followed, measuring the gap as a function of time and calculating the parameter percentage healing/wound width as follows:

$$\text{percentage ratio healing/wound width} = (width_t/width_0) \times 100$$

where $width_t$ = width of gap at time t (24, 48, 72, 98 h) and $width_0$ = width of gap at time 0 (just after the removal of the insert).

Statistical analysis

The results of the viscosity, mucoadhesion measurements and ELISA assay of GFs were analysed by means of the Mann–Whitney U test (Siphar, Creteil, F). The results of proliferation and wound-healing measurements were analysed using the Kruskal–Wallis test and Dunn's post-hoc comparison (Stata 11® Statacorp. 2009, Stata statistical software release 11, Statacorp LP, Collage Station, TX).

Results and Discussion

Rheological characterization

Figure 1 shows the viscosity curves of the sterile CSG vehicle (CSG) and the CSG formulation (PL–CSG) and Figure 2 shows the sterile PAA vehicle (PAA) and the PAA formulation (PL–PAA). Both vehicles have similar viscosity profiles and the dilution with PL to obtain the formulations caused, as

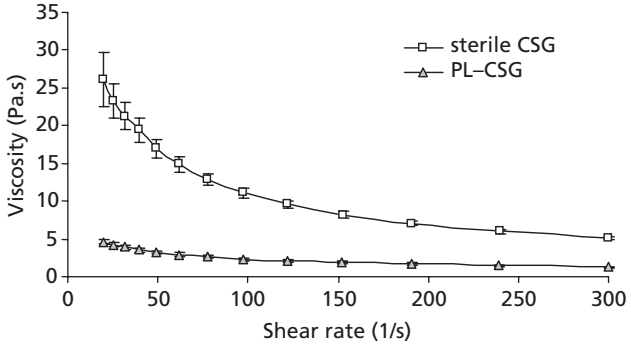


Figure 1 Viscosity curves of sterile CSG vehicle (CSG) and CSG formulation (PL–CSG) (mean values ± SD; $n = 5$).

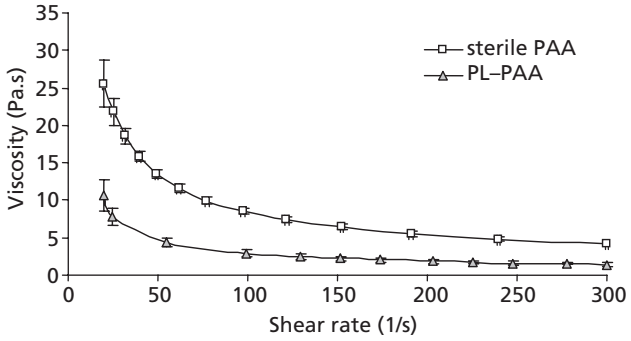


Figure 2 Viscosity curves of sterile PAA vehicle (PAA) and PAA formulation (PL–PAA) (mean values ± SD; $n = 5$).

expected, a decrease of the respective viscosity values. This is due mainly to the dilution, which determines a half polymer concentration and, moreover, especially in the case of PAA, there is also an effect due to ionic moieties of PL, which can further decrease viscosity.[28] The polymeric solutions as vehicles and after dilution, showed pseudoplastic behaviour. In fact, the viscosity decreased with increasing rate of shear stress. This is more pronounced for PL–PAA than for PL–CSG. In particular, comparing the vehicles, the percentage decrease in the viscosity of PAA was 37.9% (SD ± 0.4) while the percentage decrease in the viscosity of CSG was significantly lower at 26.7% (SD ± 2.8) ($P < 0.01$, Mann–Whitney test). In the PL formulations, the percentage decrease in the viscosity of PAA was 43.4% (SD ± 0.3) while the decrease in viscosity of CSG was significantly lower at 24.0% (SD ± 0.7) ($P < 0.01$ Mann–Whitney test). This may be due to the higher viscosity of the PL–PAA formulation compared to the PL–CSG formulation.

Since the pseudoplasticity determines a change in viscosity that occurs in response to force application, administration of the treatment by the squeezing of a bottle towards the eye surface causes the transition of the formulation to a system that flows like a liquid: in fact at higher shear rates the apparent viscosity for both samples is low, and this should make it easier to spread the sample onto the application site. Moreover, after application the relatively low shear rates should lead to higher viscosity, giving resistance towards the mechanical effects of blinking.

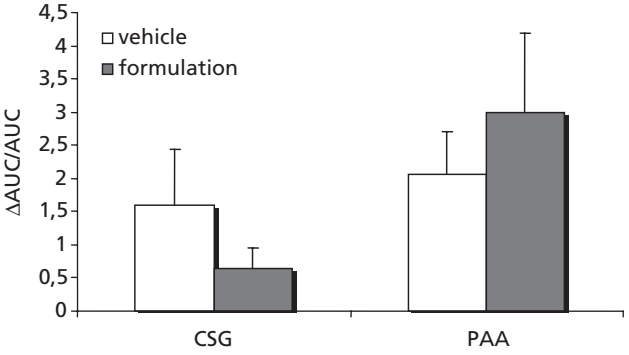


Figure 3 ΔAUC/AUC calculated for CSG and PAA vehicles and for the corresponding final formulations (mean values ± SD calculated by using rules for the propagation of error; $n = 6$).

Mucoadhesion properties

The values of the mucoadhesive parameter ΔAUC/AUC calculated for the CSG and PAA vehicles and for the corresponding final formulations PL–CSG and PL–PAA are shown in Figure 3. In all samples the parameter ΔAUC/AUC is positive, indicating that both vehicles and formulations are capable of interaction with mucin to form a strong mucoadhesive joint. This is in accordance with the well-known mucoadhesion properties of chitosan and PAA. The values of ΔAUC/AUC are similar, and not significantly different for the CSG and PAA vehicles, which therefore show comparable mucoadhesion properties (Mann–Whitney test). As for PAA and CSG, the formulations and vehicles are characterized by superimposable mucoadhesion potential: the dilution with PL does not affect the capability of the biopolymers to interact with biological substrates. The PL–PAA formulation possesses a mucoadhesive potential significantly higher than that of PL–CSG ($P < 0.01$, Mann–Whitney test). The loading of PL in polymeric vehicles does not decrease the mucoadhesive potential: the ΔAUC/AUC parameter normalizes the dilution effect (halving the polymer concentrations) by deleting the effect of consistency on mucoadesion. This aspect is particularly important since mucoadhesion is necessary to maintain and prolong the contact of the bioactive molecules and especially GFs in the PL with the corneal lesions: the mucoadhesive interaction between formulation and mucous layer should maximize PL's biochemical effect, helping the healing process.

ELISA assay of growth factors

One of the major concerns in the employment of polymeric systems in the formulation of especially complex therapeutics such as biotechnological ones is the compatibility of polymer and active agent. To this end, the quantification of PDGF-AB was performed, both on the PL and on the formulations, by means of an ELISA assay. PDGF-AB was chosen as representative of the GFs present in platelets as it is one of the better known and best-characterized GFs. Moreover, PDGF AB with TFG-β is present in the highest amounts and it is responsible for promoting the healing of soft tissue through stimulation of collagen production.[29] Because the quantification is based on the binding of the PDGF-AB present in the

samples to a specific immobilized monoclonal antibody, it can be assumed that the amount of PDGF-AB detected has maintained a structure that still allows this interaction. To perform a more precise comparison of the two polymeric systems, the percentage ratio of PDGF-AB formulation to PL parameter was calculated for both PL CSG and PL–PAA formulations. The values of this parameter are directly related to the effect of the polymer on the PDGF-AB molecular structure. The presence of chitosan or PAA did not affect PDGF-AB concentrations, which were close to 100% and not significantly different from those quantified in PL (Mann–Whitney test). In particular PL–CSG is characterized by a ratio of PDGF-AB formulation to PL of 117.5% (SD ± 11.7) while for PL–PAA, the figure was 113.5% (SD ± 5.8).

This result suggests that the presence of mucoadhesive polymers did not alter the structure of PDGF-AB and so it is conceivable that they did not alter its activity. It can be argued that the effect of the polymers on the other GFs in PL should be similar.

Proliferation on RCE cells

Given the complexity of the platelet GF pool, a characterization based on in-vitro dosage of single factors, as can be done with the ELISA test, could be meaningful and predictive of sample efficacy if a correlation between the content and pattern of GFs with hemoderivative activity is established.

In Table 1 the percentage values of 24 h cellular proliferation of RCE in the presence of PL at two different dilutions (1/20 and 1/40), and in the presence of PL at the same dilutions but mixed with the two polymers in formulations, are reported. The results of the complete statistical analysis are reported under the table. The same batches of PL were used to compare PL activity with and without polymers, although different batches were used for the two series of experiments. Both PL alone and PL in formulation (PL–CSG and PL–PAA) had been maintained at 4°C for different times (time zero T0, 7 days T7, 10 days T10, 15 days T15) before evaluation. The proliferation obtained in complete culture medium (with FCS and EGF) is considered 100%. 'Control' refers to the percentage proliferation obtained with the medium without FCS and EGF. It is therefore possible to compare the proliferation induced by the GFs contained in the PL with that obtained at the same times in the controls grown without GFs. In both series (Table 1), in spite of the natural variability in GF content and the differences between PL pools, this comparison showed that a significant positive effect on proliferation was obtained with PL samples at the two different dilutions at every time point considered. This can be clearly observed at all the time points considered up to 15 days, indicating good stability of the PL at 4–8°C.

In the PL–CSG formulation (Table 1) there was a significant increase in proliferation for the 1/20 dilution with respect to the control at all time points considered: this indicates that the PL contained in the CSG vehicle was able to maintain its proliferative activity for up to 15 days. This result is of particular importance when patients are treated with autologous PL, as they can obtain the formulation and keep it in the fridge at home for at least 2 weeks before coming back to the hospital to receive another preparation.

In the case of the PAA formulation (Table 1), again the stability of the PL is confirmed for up to 15 days, although in this case the proliferation was in some cases lower than 100% and significantly different to the control. In a previous study of ours, a formulation based on PL–PAA was tested *in vivo* on a small panel of patients, with positive effects on buccal mucosa repair.[18] The same formulation was also tested *in vitro* for cell proliferation of fibroblasts, which are among the first choice cell culture models for proliferation assays.[30] To better evaluate the results, in Table 2 a comparison is given of the results obtained with PL–CSG and with PL–PAA on RCE, and with PL–PAA on fibroblasts, by using as a parameter the ratio between the proliferation induced by the formulations and that induced by the PL apheresis sample that was used to prepare the same formulations. This parameter has the advantage of reducing the effect of the intrinsic variability of PL pools. The results of the complete statistical analysis are reported under the table. The PL–PAA formulation at 1/40 PL concentration was able to increase the proliferation of fibroblasts at all times up to 10 days at a comparable extent to the CSG formulation on corneal cells (RCE). However, the results obtained from treating the RCE substrate with the PAA formulation were less encouraging than those obtained using CSG one. This could be attributed to the polymer effect or to a higher sensitivity of the RCE cell line or to a combination of both these effects. Even if chitosan shows a promising in-vitro effect, its formulations produce a proliferation about half that of PL. This indicates that both the polymers used impair cell growth in the experimental conditions of the test to some extent. However, the normalization of proliferation with respect to PL performance showed no significant differences between the proliferations induced by CSG and PAA on both cell lines at 40-fold dilution. At 20-fold dilution it can be argued that PAA had the same proliferation effect on both cell lines, and that CSG was able to enhance cell growth to a greater extent than PAA at 7, 10 and 14 days of storage: in particular at 7 and 10 days of storage the effect of CSG is higher than that of PL as indicated by a ratio higher than 1. These results suggest that chitosan is characterized by intrinsic proliferation properties. Moreover RCE cells are particularly sensitive to the action of proliferation enhancers and GFs. In particular the growth of this cell line is strictly related to the presence of EGF, whose role has been described as important in accelerating proliferation of corneal epithelial cells and also migration of corneal epithelial cells *in vivo*.[19]

In-vitro wound-healing test

Fibroblasts have a crucial role in corneal healing: after a wound occurs, within the stroma, activated fibroblasts appear in response to injury.[31] Moreover, fibroblasts migrate to the wounded area, secreting collagenases, proteases and extracellular matrix components, which contribute to the reconstruction of damaged stroma.[22]

Figure 4 shows the values of the percentage ratio of healing/wound width calculated for PL, for PL–CSG and PL–PAA at 24, 48, 72 and 96 h (the dotted line is 100% – the initial gap width). The results of the complete statistical

Table 1 Values of 24 h cellular proliferation of RCE in presence of PL or PL formulations

		CSG		
Time (days)	0	7	10	15
Control	72.1 ± 6.0	109.7 ± 15.8	95.8 ± 14.3	103.4 ± 8.2
PL 1/20	543.1 ± 46.2	303.6 ± 27.0	325.3 ± 28.6	461.0 ± 40.2
PL 1/40	559.9 ± 33.3	359.0 ± 38.3	310.1 ± 35.4	433.2 ± 65.8
PL CSG 1/20	226.1 ± 19.8	484.9 ± 71.6	340.5 ± 33.4	279.5 ± 34.6
PL CSG 1/40	265.0 ± 16.4	180.1 ± 22.2	192.9 ± 18.8	214.8 ± 27.0
		PAA		
Time (days)	0	7	10	15
Control	122.3 ± 7.6	55.8 ± 2.8	70.8 ± 3.1	102.0 ± 8.5
PL 1/20	317.4 ± 14.9	373.9 ± 48.4	379.7 ± 16.6	501.6 ± 32.3
PL 1/40	386.5 ± 15.2	226.7 ± 12.7	392.8 ± 19.0	595.6 ± 31.3
PL–PAA 1/20	103.7 ± 3.9	150.0 ± 21.5	81.3 ± 4.8	110.7 ± 6.1
PL–PAA 1/40	93.7 ± 4.3	102.4 ± 13.5	88.1 ± 4.3	92.2 ± 4.0

Mean values ± SE; $n = 32$. The results of the complete statistical analysis are reported below.
Statistical evaluation (post hoc Dunn's test, Kruskal Wallis test)
CSG
$t = 0$
control vs PL 1/20 $P < 0.001$
control vs PL 1/40 $P < 0.001$
control vs PL CSG 1/20 $P = 0.004$
control vs PL CSG 1/40 $P < 0.001$
PL 1/20 vs PL 1/40 $P = 0.371$
PL 1/20 vs PL CSG 1/20 $P < 0.001$
PL 1/40 vs PL CSG 1/40 $P < 0.001$
PL CSG 1/20 vs PL CSG 1/40 $P = 0.279$
$t = 7$
control vs PL 1/20 $P < 0.001$
control vs PL 1/40 $P < 0.001$
control vs PL CSG 1/20 $P < 0.001$
control vs PL CSG 1/40 $P = 0.059$
PL 1/20 vs PL 1/40 $P = 0.200$
PL 1/20 vs PL CSG 1/20 $P = 0.194$
PL 1/40 vs PL CSG 1/40 $P < 0.001$
PL CSG 1/20 vs PL CSG 1/40 $P < 0.001$
$t = 10$
control vs PL 1/20 $P < 0.001$
control vs PL 1/40 $P < 0.001$
control vs PL CSG 1/20 $P < 0.001$
control vs PL CSG 1/40 $P = 0.009$
PL 1/20 vs PL 1/40 $P = 0.212$
PL 1/20 vs PL CSG 1/20 $P = 0.469$
PL 1/40 vs PL CSG 1/40 $P = 0.015$
PL CSG 1/20 vs PL CSG 1/40 $P = 0.001$
$t = 14$
control vs PL 1/20 $P < 0.001$
control vs PL 1/40 $P < 0.001$
control vs PL CSG 1/20 $P < 0.001$
control vs PL CSG 1/40 $P = 0.008$
PL 1/20 vs PL 1/40 $P = 0.215$
PL 1/20 vs PL CSG 1/20 $P = 0.008$
PL 1/40 vs PL CSG 1/40 $P = 0.004$
PL CSG 1/20 vs PL CSG 1/40 $P = 0.160$
PAA
$t = 0$
control vs PL 1/20 $P < 0.001$
control vs PL 1/40 $P < 0.001$
control vs PL–PAA 1/20 $P = 0.002$
control vs PL–PAA 1/40 $P = 103$
PL 1/20 vs PL 1/40 $P = 0.292$
PL 1/20 vs PL–PAA 1/20 $P < 0.001$
PL 1/40 vs PL–PAA 1/40 $P < 0.001$
PL–PAA 1/20 vs PL–PAA 1/40 $P = 0.051$
$t = 7$
control vs PL 1/20 $P < 0.001$
control vs PL 1/40 $P < 0.001$
control vs PL–PAA 1/20 $P < 0.001$
control vs PL–PAA 1/40 $P = 0.002$
PL 1/20 vs PL 1/40 $P = 0.005$
PL 1/20 vs PL–PAA 1/20 $P < 0.001$
PL 1/40 vs PL–PAA 1/40 $P = 0.017$
PL–PAA 1/20 vs PL–PAA 1/40 $P = 0.240$
$t = 10$
control vs PL 1/20 $P < 0.001$
control vs PL 1/40 $P < 0.001$
control vs PL–PAA 1/20 $P = 0.281$
control vs PL–PAA 1/40 $P = 0.195$
PL 1/20 vs PL 1/40 $P = 0.011$
PL 1/20 vs PL–PAA 1/20 $P < 0.001$
PL 1/40 vs PL–PAA 1/40 $P < 0.001$
PL–PAA 1/20 vs PL–PAA 1/40 $P = 0.390$
$t = 14$
control vs PL 1/20 $P < 0.001$
control vs PL 1/40 $P < 0.001$
control vs PL–PAA 1/20 $P = 0.013$
control vs PL–PAA 1/40 $P = 0.104$
PL 1/20 vs PL 1/40 $P = 0.217$
PL 1/20 vs PL–PAA 1/20 $P < 0.001$
PL 1/40 vs PL–PAA 1/40 $P < 0.001$
PL–PAA 1/20 vs PL–PAA 1/40 $P = 0.171$

Table 2 Ratio between proliferation induced by the formulations and that induced by the corresponding PL

Ratio form/PL (1/20)				
Time (days)	0	7	10	14
CSG RCE 1/20	0.364 ± 0.051	1.681 ± 0.418	1.098 ± 0.166	0.452 ± 0.095
PAA RCE 1/20	0.604 ± 0.066	0.391 ± 0.074	0.292 ± 0.027	0.330 ± 0.074
PAA fibro 1/20	0.378 ± 0.073	0.814 ± 0.266	0.372 ± 0.079	0.381 ± 0.128
Ratio form/PL (1/40)				
Time (days)	0	7	10	14
CSG RCE 1/40	0.490 ± 0.063	0.404 ± 0.053	0.528 ± 0.098	0.484 ± 0.101
PAA RCE 1/40	0.437 ± 0.054	0.696 ± 0.148	0.377 ± 0.052	0.218 ± 0.021
PAA fibro 1/40	0.619 ± 0.074	1.259 ± 0.266	0.382 ± 0.066	0.709 ± 0.127

Comparison of the results obtained with PL–CSG and with PL–PAA on RCE and with PL–PAA on fibroblasts. The results of the complete statistical analysis are reported below.
Statistical evaluation (post hoc Dunn's test, Kruskal–Wallis test)
Ratio form/PL (1/20)
$t = 0$ overall $P = 0.327$
$t = 7$
CSG RCE 1/20 vs PAA RCE 1/20 $P < 0.001$
CSG RCE 1/20 vs PAA fibro 1/20 $P < 0.001$
PAA RCE 1/20 vs PAA fibro 1/20 $P = 0.457$
$t = 10$
CSG RCE 1/20 vs PAA RCE 1/20 $P < 0.001$
CSG RCE 1/20 vs PAA fibro 1/20 $P < 0.001$
PAA RCE 1/20 vs PAA fibro 1/20 $P = 0.500$
$t = 14$
CSG RCE 1/20 vs PAA RCE 1/20 $P = 0.005$
CSG RCE 1/20 vs PAA fibro 1/20 $P = 0.005$
PAA RCE 1/20 vs PAA fibro 1/20 $P = 0.500$
Ratio form/PL (1/40)
$t = 0$ overall $P = 0.997$
$t = 7$ overall $P = 0.299$
$t = 10$ overall $P = 0.443$
$t = 14$
CSG RCE 1/20 vs PAA RCE 1/20 $P = 0.001$
CSG RCE 1/20 vs PAA fibro 1/20 $P < 0.001$
PAA RCE 1/20 vs PAA fibro 1/20 $P = 0.001$

analysis are reported under the figure. PL and PL–CSG have similar values of the ratio, close to 70% after 24 h, indicating that about 25% of the gap has been covered by cells. After only 48 h, the gap cannot be measured any longer, either for PL or PL–CSG. As for PL–PAA, a first significant reduction of the gap could be appreciated only after 48 h, and then the gap decreased quite linearly as a function of time: at 96 h it was close to 50%. Figure 5 shows microphotographs of the gaps in fibroblast substrates after 48 h of contact with: (a) PL at 1/20 dilution, (b) PL–CSG at a PL concentration of 1/20 and (c) PL–PAA at a PL concentration of 1/20. In the case of PL and CSG (Figure 5a and b, respectively), at 48 h the invasion of the fibroblasts is complete and a gap cannot be seen any more. In the case of PAA the proliferation occurs slowly and the gap is still visible after 48 h. In all substrates, fibroblasts appear fusiform and no signs of apoptotic or dead cells (round-shaped cells detached from the Petri bottom) can be seen: this is also an index of the biocompatibility of both vehicles (PAA and CSG) with biological substrates.

Conclusions

The rheological properties of formulations based on chitosan or PAA indicate potentially good resistance towards the mechanical effect of blinking. The decrease in rheology after loading of PL in polymeric vehicles was due to the halving of polymer concentrations. However, the maintenance of pseudoplastic behaviour and of mucoadhesion properties may be an indicator of lack of alteration in the intrinsic polymer properties.

The mucoadhesive properties of chitosan and PAA were not impaired by the dilution of the vehicles with PL. This is an important point since mucoadhesion is considered necessary to maintain and prolong the contact of bioactive molecules and especially GFs of PL with corneal lesions, thus maximizing PL's biochemical effect.

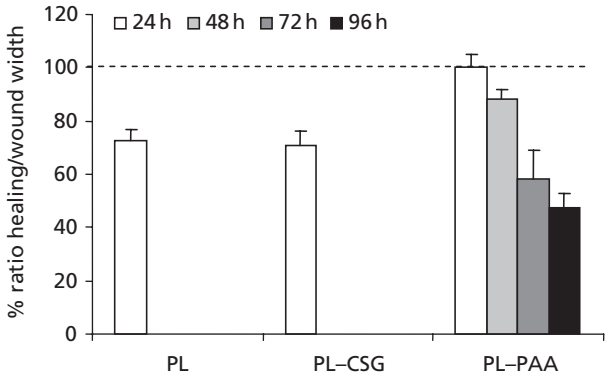
Moreover, mucoadhesive polymers were demonstrated to be compatible with PDGF-AB, as they did not alter its response to immunoassay, and conceivably its structure: this

is a confirmation of the absence of interactions between polymer chains and PL proteins.

The proliferation tests on epithelial cells demonstrate that the formulation based on CSG and PL is able to significantly enhance cell growth on storage times of up to 2 weeks, while the formulation based on PAA and PL was less efficient in enhancing proliferation, probably also due to the characteristics of the cell culture model used. These results also show the proliferation enhancing effect of chitosan.

The in-vitro wound-healing test revealed that the two polymers caused clear differences in the rate of fibroblast proliferation. The effects induced by PL and the PL–CSG formulation were faster and the in-vitro wound was filled by cells after 48 h. The same result was obtained with the PL–PAA formulation, but only after 96 h. This kinetic difference should be taken into account according to the target tissue. In the case of buccal application, as was demonstrated in our previous studies,[17,18] the slowest action of PL–PAA formulation can result in the desired therapeutic effect, while the shorter contact time typical of ocular applications would point the choice towards the employment of chitosan.

The formulations proposed based on a biopolymer vehicle loaded with PL, rich in GFs, should have a great impact on repair medicine and in particular on the therapy of chronic ophthalmic lesions since, until now, the current medical and surgical techniques proposed are not resolutive and the management of these conditions remains problematic and healing with standard protocols is unattainable. The rheological and mucoadhesive properties of vehicles combined with proliferation and wound healing capability should ensure a regenerative potential which will trigger the healing process, due to the high concentration of GFs: these start and amplify the lesion resolution, stimulating effects such as angiogenesis, chemotaxis of macrophages, proliferation and migration of fibroblasts, and the synthesis of collagen.



Statistical evaluation (post hoc Dunn's test, Kruskal Wallis test)
t=24 h
PL vs PL PAA p<0.001
PL vs PL CSG p=0.48
PL PAA vs PL CSG p<0.001

t=48 h
PL vs PL PAA p<0.001
PL vs PL CSG p=0.50
PL PAA vs PL CSG p<0.001

t=72 h
PL vs PL PAA p<0.001
PL vs PL CSG p=0.50
PL PAA vs PL CSG p<0.001

t=96 h
PL vs PL PAA p<0.001
PL vs PL CSG p=0.50 (NS)
PL PAA vs PL CSG p<0.001

Figure 4 Ratio healing/wound width calculated for PL, for PL–CSG and PL–PAA at 24, 48, 72 and 96 h (dotted line is 100% that is gap width at time 0) (mean values ± SD; $n = 24$). The results of the complete statistical analysis are reported under the figure.

Conflict of interest

The author(s) declare that they have no conflicts of interest to disclose.

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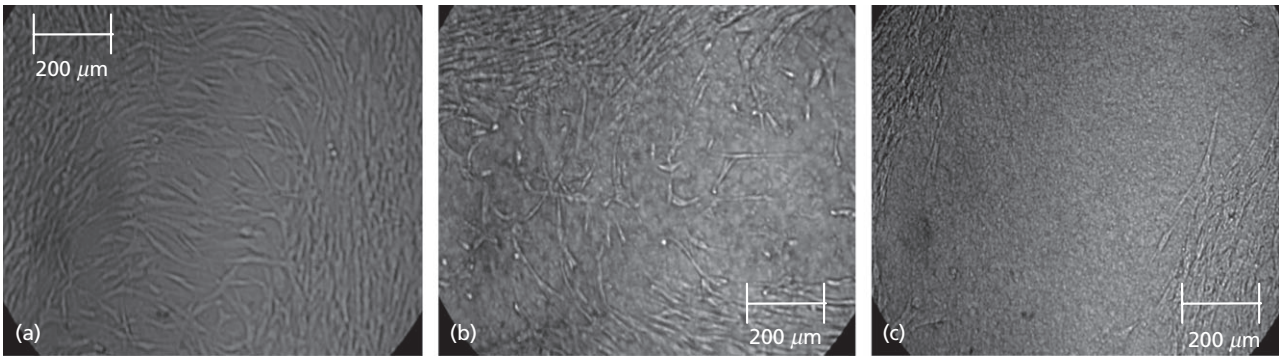

Figure 5 Microphotographs of gaps in fibroblast substrates after 48 h of contact with: (a) PL at 1/20 dilution, (b) PL CSG at PL dilution of 1/20, (c) PL–PAA at PL dilution of 1/20 (mean values ± SD; $n = 16$).

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