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A novel dressing for the combined delivery of platelet lysate and vancomycin hydrochloride to chronic skin ulcers: Hyaluronic acid particles in alginate matrices

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ABSTRACT

The aim of the present work was to develop a medication allowing for the combined delivery of platelet lysate (PL) and an anti-infective model drug, vancomycin hydrochloride (VCM), to chronic skin ulcers. A simple method was set up for the preparation of hyaluronic acid (HA) core-shell particles, loaded with PL and coated with calcium alginate, embedded in a VCM containing alginate matrix. Two different $CaCl_2$ concentrations were investigated to allow for HA/PL core-shell particle formation. The resulting dressings were characterized for mechanical and hydration properties and tested *in vitro* (on fibroblasts) and *ex-vivo* (on skin biopsies) for biological activity. They were found of sufficient mechanical strength to withstand packaging and handling stress and able to absorb a high amount of wound exudate and to form a protective gel on the lesion area. The $CaCl_2$ concentration used for shell formation did not affect VCM release from the alginate matrix, but strongly modified the release of PGFAB (chosen as representative of growth factors present in PL) from HA particles. *In vitro* and *ex vivo* tests provided sufficient proof of concept of the ability of dressings to improve skin ulcers healing.

1. Introduction

In the last decades, the therapeutic approaches for the management of difficult-to-treat wounds have undergone progressive change: “inert” dressings, having the unique aim of isolating the lesion area after its disinfection, have been replaced by “bioactive” dressings, based on biopolymers able to interact with tissue components and take part in the healing process (Rossi et al., 2007; Rossi et al., 2015; Caramella et al., 2016; Mori et al., 2016a; Francesko et al., 2017; Rossi et al., 2017; Tenci et al., 2017a, 2017b;Pallavicini et al., 2017; Sandri et al., 2017).

Hyaluronic acid (HA), also known as hyaluronan, is predominantly present in the extracellular matrix and found at high concentration in human umbilical cord, synovial fluid, connective tissue, vitreous body and skin (Fraser et al., 1997). HA is synthetized in high quantities by fibroblasts (Belvedere et al., 2017) and is involved in all stages of wound healing process with different mechanisms (Chen and Abatangelo, 1999). Some authors have shown that low molecular weight (MW) HA fragments (100 to 300 kDa) promote wound repair and increase the self-defense of skin epithelium (Gariboldi et al., 2008; Ghazi et al., 2012). Moreover, it is reported that HA actively participates in the regulation of skin cell proliferation by interacting with CD44 receptor, which is expressed in the majority of skin cells, including fibroblasts and keratinocytes (Banerji et al., 2007; Kaya et al., 2006).

It is well known that growth factors are key biological mediators of the healing process (Werner and Grose, 2003). Among these, platelet derived growth factor (PDGF) is one of the most extensively investigated. Recently, Li et al. (2017) developed a polymeric sponge able to sequester PDGF BB at the site of injury and to create a suitable niche which facilitates endogenously-driven healing process. Such a system, based on a galacturonic acid-containing polysaccharide, selectively binds endogenous PDGF BB and manages its release in response to the progression of wound healing. This facilitates PDGF BB-mediated cellular functions, resulting in improved wound healing due to controlled immune activation and enhanced neovascularization and new tissue formation.

In recent years, some authors suggested the therapeutic use of hemoderivatives, in particular of platelet lysate (PL), to enhance wound healing (Crovetti et al., 2004; Ranzato et al., 2008). PL, which is rich in growth factors and other biologically active substances, proved capable of promoting tissue regeneration. More recently, Sergeeva et al. (2016)

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investigated the reparative activity of PL, analyzing its effect both *in vitro* on cell monolayer recovery and *in vivo* on skin wound healing. They proved that PL stimulates epithelialization of skin defects and accelerates granulation tissue formation. Along this line different formulations loaded with PL and intended to treat skin lesions have been developed and tested for wound healing enhancement (Rossi et al., 2013; Dellera et al., 2014; Mori et al., 2014; Sandri et al., 2015; Mori et al., 2016a, 2016b; Tenci et al., 2016).

A major problem associated with chronic wounds is that they are prone to infections, which are the most likely single cause of delayed healing. Infections, if untreated, can progress from local to systemic ones and result in sepsis and multiple organ dysfunction syndrome (Leaper et al., 2015), which makes loading of anti-infective drugs in wound dressings a requirement.

Given these premises, the aim of the present work was to develop a dressing for the combined delivery of platelet lysate (PL) and an anti-infective model drug, vancomycin hydrochloride (VCM), to chronic skin ulcers. Dressings should be easy to prepare in view of their employment in a hospital pharmacy service, where autologous hemoderivative samples (directly prepared from the patient to be treated) can be used. The formulation should also allow for keeping the two actives physically separated and avoid incompatibility problems. The strategy used was to separately load PL into hyaluronic acid (HA) core –shell particles coated with calcium alginate, which then resulted embedded in the alginate (ALG) containing vancomycin (VMC). The underlying idea was that VCM would protect the lesion against bacterial infections, whereas HA and PL would act as healing enhancers.

The research project was developed in two phases. In the first one, the dressings were prepared and characterized for mechanical, hydration and rheological properties. Dressings were prepared by dropping an aqueous mixed solution containing HA/PL/$CaCl_2$ into an alginate solution containing VCM, subsequently freeze-drying the resultant dispersion. Two different $CaCl_2$ concentrations were used to allow for different extent of cross-linking.

Dressings should possess suitable mechanical properties to resist stresses during packaging and administration. Moreover, they should absorb excess exudate and form a gel, having rheological properties functional to the protection of the lesion area.

The second phase of the research focused on checking the biological properties. Dressings were characterized for VCM and PDGF AB (platelet derived growth factor) loading capacity. PDGF AB was chosen as representative of the pool of growth factors present in PL. VCM and PDGF AB release was assessed *in vitro*. Wound healing properties were investigated *in vitro* on human dermal fibroblasts and *ex vivo* on human skin biopsies.

2. Materials and methods

2.1. Materials

The following materials were used: Acetonitrile (Carlo Erba, Milan, I); Alginic acid sodium salt (ALG), from brown algae, medium viscosity grade (MV) (~2000 cps) (Sigma Chimica, Milan, I); Hyaluronic acid sodium salt (HA) low MW (Bioiberica, Barcelona, S); Anti-mouse IgG-FITC antibody (Sigma Aldrich, Milan, I); 5-Bromo-2′deoxyuridine (BrdU) (Sigma Aldrich, Milan, I); $CaCl_2$ (Sigma Chimica, Milan, I); Dulbecco's Modified Eagles Medium (DMEM, Lonza, BioWhittaker, B); Dulbecco's Phosphate Buffer Solution (Sigma Aldrich, Milan, I); Hank's Balance Salt Solution (HBSS) (Sigma Aldrich, Milan, I); Hoechst 33258 (Sigma Aldrich, Milan, I); Inactivated foetal calf bovine serum (Euroclone, Pero, I); Mouse anti-Bromo-deoxyuridine antibody (anti-BrdU antibody) (GE Healthcare UK Ltd., Amersham Place, Buckinghamshire, England); Mowiol (Sigma Aldrich, Milan, I); MTT (3-(4,5- methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma Aldrich, Milan, I); $NaH_2PO_4{\cdot}H_2O$ (Carlo Erba, Milan I); $Na_2HPO_4{\cdot}H_2O$ (Carlo Erba, Milan, I); NaCl (Carlo Erba, Milan, I); Penicillin streptomycin 100× (pen/strep)/amphoteric 100× (Sigma Aldrich, Milan, I); PTA-blocking solution (PBS/Tween20/Albumin) (Sigma Aldrich, Milan, I); Trypan Blue solution (Biological Industries, Beit-Haemek, IL); Trypsin-EDTA solution (Sigma Aldrich, Milan, I).

Platelet lysate was supplied by Immunohaematology and Transfusion Service and Cell Therapy Unit of Fondazione IRCCS, S. Matteo, Pavia, I. A pooled sample prepared from platelet rich plasma (containing 500 × 10^3 platelets/μl) obtained from eight different blood donors was used.

2.2. Dressing preparation

Dressings were prepared by dropping with a 32G needle (mesorelle®, Aiesi Hospital Service, Naples, I) 1 ml of 1: 1 w/w mixture of 6% w/w HA aqueous solution containing 2 or 4% w/w $CaCl_2$ and PL in 50 ml of 1% w/w sodium alginate (ALG) aqueous solution containing VCM (3 mg/ml) that was maintained under gentle stirring for 30 min. The final $CaCl_2$ concentrations into HA/PL mixtures were 1 and 2% w/w. Then, a portion of ALG solution was removed to obtain 8 g of formulation, that were congealed at −40 °C and, then, freeze-dried (Heto DRY WINNER®, Analitica De Mori, Milan, I) to produce circular dressing with a diameter of 40 mm and the following thicknesses: 6.2 (± 0.59) mm in presence of $CaCl_2$ 1% w/w and 7.2 (± 0.67) mm in presence of $CaCl_2$ 2% w/w (n = 3). HA particles had a diameter of 1.9 (± 0.2) mm in presence of 1% w/w $CaCl_2$ and of 2.2 (± 0.1) in presence of 2% w/w $CaCl_2$ (n = 3). Fig. 1 reports, as an example, a picture of the dressing, dried (A) and hydrated (C), and of HA particles (B) prepared in presence of 1% $CaCl_2$ concentration.

Unloaded dressings were also prepared according to the same procedure employed for loaded ones, substituting PL and VCM with distilled water.

2.3. Dressing characterization

2.3.1. Tensile measurements

Dressings were placed in an air-tight container at 25 °C in presence of a saturated NaCl solution in order to reach 10% water content, which, acting as plasticizer, improves their mechanical properties (Rossi et al., 2013). Then, dressings were subjected to tensile measurements by mean of TA.XT plus apparatus (ENCO, Spinea, I), equipped with the A/TG Tensile Grips measuring system. According to

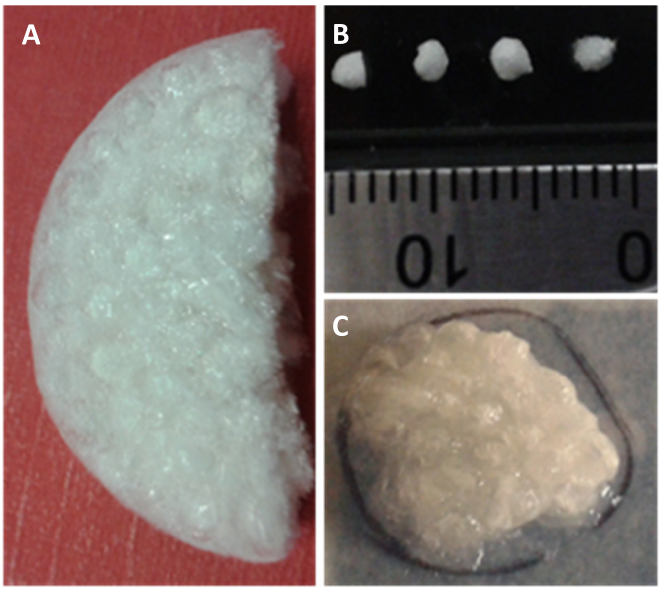


Fig. 1. Picture of a dressing prepared with 1% w/w $CaCl_2$ concentration (dry (A) and hydrated (C)) and of HA particles (B).

Mori et al. (2016a) a portion of dressing (20 mm × 20 mm) was kept vertical by means of 2 jaws (the lower one fixed and the upper one movable). The movable jaw was raised at a constant rate (1 mm/s) up to dressing fracture and the relevant fracture force (mN) and distance (mm) were recorded. The parameter fracture work was also calculated as the area under the curve force *vs* distance. Elongation at break (%E) was determined using the following equation (Rezvanian et al., 2017):

$$\%E = (\text{extension of length at rupture/initial length}) * 100$$

Three replicates were performed for each dressing.

2.3.2. Hydration measurements

Dressings were subjected to hydration measurements at 32 °C by means of Franz diffusion cell (PermeGear, Bethlehem (PA)). In particular, a weighted portion of dressings (20 mm × 20 mm) was layered on a dialysis membrane in the apical chamber of the Franz cell. The receptor chamber was filled with PBS buffer (pH 7.4), to mimic wound exudate. Dressing portion was weighted after 6 h and 18 h contact with PBS solution. Dressing hydration propensity was evaluated as the amount of PBS absorbed, normalized by dressing weight.

2.3.3. Rheological properties

After 6 h and 18 h hydration, dressings were subjected to viscosity and viscoelastic measurements by means of a rotational rheometer (Rheostress 600, Haake, I) equipped with a cone plate combination (C20/4: diameter = 20 mm; angle = 4°) measuring system. All measurements were carried out at 32 °C after a rest time of 3 min. Apparent viscosity was measured at increasing shear rate values ranging into 10–300 s^{-1} interval. Dynamic viscoelastic measurements were also effected. In particular, stress sweep and oscillation tests were performed. In the stress sweep test shear stress values increasing into the interval 0.004–10 Pa were applied at a constant frequency (0.1 Hz) and the elastic response of the sample, expressed as storage modulus G′, was measured. Such a test allows for identifying the “linear viscoelastic region”. In the oscillation test a shear stress, chosen in the linear viscoelastic region previously identified, was applied at increasing frequency values (0.1–10 Hz) and the elastic and viscous response of the sample was measured as a function of frequency.

2.3.4. VCM loading capacity

An accurately weighted portion of dressing was dissolved in distilled water and analyzed by an HPLC method (Perkin Elmer® series 200; Perkin Elmer® Instruments, Sheldon, USA). The experimental conditions were: Hypersil BDS C18 (5 µm, Sheldon HPLC, Cheshire, UK) column, 90/10 pH 5 phosphate buffer (USP)/acetonitrile (Carlo Erba, Milan, I) mobile phase, 20 µl loop, 1.5 ml/min flux, lambda: 230 nm.

VCM loading capacity was calculated according to the following equation:

$$\text{Loading capacity} = (\text{VCM amount (mg) into dressing} / \text{weight (mg) of dressing}) \times 100$$

2.3.5. PL loading capacity

The concentration of the platelet derived growth factor (PDGF AB) in fresh PL and in loaded HA particles was assessed by means of ELISA test (Human PDGF AB Quantikine PharmPak, R&D Systems, Minneapolis, MN, USA; assay range: 31.2–2000 pg/ml). PDGF AB growth factor was chosen as a representative of the pool of growth factors present in PL.

In particular, HA particles (30 mg) were dissolved in 1.5 ml of 55 mM sodium citrate (pH 6.8) solution, prepared in NaCl 0.9% w/w, to allow for breaking of particle shells and PL release. Fresh PL diluted in NaCl 0.9% w/w and in 55 mM sodium citrate (pH 6.8) solution prepared in NaCl 0.9% w/w were used as references.

PDGF AB loading capacity was calculated according to the following equation:

$$\text{Loading capacity} = (\text{PDGF AB total amount (mg) in the dressing} / \text{weight (mg) of the dressing}) \times 100$$

2.3.6. Cell proliferation measurements

NHDF fibroblasts (juvenile fibroblasts from foreskin) (Promocell GmbH, Heidelberg, G) from 6 to 16th passage were used. Cells were cultured in a polystyrene flask (Greiner bio-one, PBI International, Milan, Italy) with 15 ml of complete culture medium (CM), consisting of Dulbecco's Modified Eagles Medium with 4.5 g/l glucose and L-Glutamine (DMEM, Lonza, BioWhittaker, B) supplemented with 1% (v/v) penicillin streptomycin 100× (pen/strep) 1% (v/v), amphoteric 100× (Sigma Aldrich, Milan, I) and 10% (v/v) inactivated foetal calf bovine serum (Euroclone). Cells were maintained at 37 °C in 95% air and 5% CO_2 atmosphere (Shellab® Sheldon® Manufacturing Inc., Oregon, USA). All the operations required for cell cultures were carried out in a vertical laminar air-flow hood (Ergosafe Space2, PBI International, Milan, I). After cells had reached confluence (one week), trypsinization was performed: at first the monolayer was washed with Dulbecco's Phosphate Buffer Solution (PBS) (Sigma Aldrich, Italy) in order to remove bivalent cations that could inactivate trypsin, then 3 ml of 0.25% (v/v) trypsin-EDTA solution (Sigma Aldrich, Milan, I) were left in contact with the monolayer for 5 min in the incubator. Afterwards, the cell layer was harvested with 7 ml of the complete medium to stop the proteolytic activity of trypsin and to facilitate the detachment of cells. Then, cells suspension was centrifuged (Sorvall® TC6, Sorvall Products, Newtown, USA) a 112 *g* for 10 min. The supernatant was eliminated and then cells were re-suspended in 6 ml medium without serum (M w/s). The amount of cells in suspension was determined in a counting chamber (Hycor Biomedical, Garden Grove, California, USA), using a 0.5% (w/v) Trypan Blue solution (Biological Industries, Israel) to visualize cells.

Cell proliferation tests were performed by means of a Transwell® Permeable Supports system (Corning Costar, USA) (Mori et al., 2016b). In particular, cells were seeded in the basolateral chamber at 60,000 cell/cm^2 in presence of medium without serum (M w/s), then a weighted portion of freeze-dried formulation was placed onto the filter of the apical chamber. Dressings loaded and unloaded with PL obtained with both $CaCl_2$ concentrations were hydrated with 500 µl of HBSS (pH 7.4) and left in contact with cells for 72 h, in order to release the active substances. After that time, 500 µl of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) 8 µM in 1000 µl of HBSS (pH 7.4) were added to each well and incubated for 4 h. After 60 s shaking, the solution absorbance was determined at a wavelength of 570 nm, with a 690 nm reference wavelength, by means of an IMark®Microplate reader (Bio-Rad Laboratories S.r.l., Segrate, Milan, I). Results were expressed as % proliferation, normalizing absorbance measured in presence of dressings with that measured after contact with M w/s, fresh PL and freeze dried PL.

2.3.7. DNA synthesis by BrdU incorporation

To verify the proliferative properties of cells after contact with dressings, the DNA synthesis was analyzed by measuring Bromo-deoxyuridine (BrdU) incorporation in all cells grown on coverslips as described in Mori et al. (2014). Briefly, 5×10^4 cells were seeded on a coverslip (Ø = 13 mm) and put in the basolateral chamber of Transwell® Permeable Supports system (Corning Costar, USA) in the presence of a medium without serum (M w/s); meanwhile a weighted portion of dressing was placed onto the filter of the apical chamber. Dressing was hydrated with 500 µl of HBSS and left in contact with cells for 72 h. During the last hour in the culture, the cells were labelled by adding 30 µM BrdU (Sigma-Aldrich, Milan, Italy) to the buffer; then cells were washed in PBS and fixed in 70% ethanol. Incorporated BrdU was detected by immunostaining reaction with anti-BrdU antibody (GE

Healthcare UK Ltd., Amersham Place, Buckinghamshire, England). Coverslips were washed with PBS and incubated with HCl 2 N for 30 min at room temperature to hydrolyze the DNA molecule and then with 0.1 M sodium tetraborate (pH 8.5) for 15 min to neutralize the solution. Samples were washed twice for 5 min in PBS and incubated for 20 min in a PTA blocking solution (1% w/v BSA and 0.02% w/v Tween20 in PBS). Cells were then incubated for 1 h with mouse anti-BrdU antibody, diluted 1:100 w/v in PTA solution, washed 3 times (10 min each) with the same solution, and then incubated for 30 min in PTA solution containing anti-mouse IgG FITC-antibody (Sigma-Aldrich, 1:100 w/v dilution). Finally, coverslips were again extensively washed with PBS, counterstained for DNA with 0.5 μg/ml Hoechst 33258 (Sigma-Aldrich), and mounted in Mowiol (Sigma-Aldrich, Milan, I). Cells were scored for BrdU immunofluorescence positivity with a Zeiss Axiophot fluorescence microscope (Carl Zeiss, Oberkochen, G). Each experiment was repeated 3 times and at least 500 cells were counted for each condition.

2.3.8. VCM release properties

VCM release properties were evaluated at 32 °C by means of Franz diffusion cells employing PBS buffer (pH 7.4) as receptor medium. In particular a weighted portion of dressing (0.021 ± 0.002 g for dressing prepared with 1% w/w $CaCl_2$; 0.033 ± 0.003 g for dressing prepared with 2% w/w $CaCl_2$; n = 3) was layered on the donor chamber of the cell (donor chamber hole Ø: 2 cm). The receptor medium was continuously stirred at 60 rpm and thermostated at 32 °C with a circulating jacket. At fixed time intervals (1-2-4-6 h), 500 μl of sample were collected from the receptor compartment and replaced with an equal volume of fresh buffer. The amount of VCM released was evaluated by means of a HPLC method (Perkin Elmer®series 200; Perkin Elmer®Instruments, Sheldon, USA) as described in the Section 2.3.4

2.3.9. PDGF AB release properties

Dressings were put in contact with 9.5 ml of PBS in a shaking bath (20 rpm) at 32 °C. After 2 and 6 h, 500 μl of PBS were withdrawn and replaced with fresh buffer solution. The concentration of PDGF AB in the medium was assessed by means of ELISA test as described in the Section 2.3.5.

2.3.10. Ex vivo tests on human skin biopsy

To analyze the effects of dressings on wound healing, an *ex vivo* human skin model was used to reproduce the physiological conditions (Bonferoni et al., 2018). Adult healthy human skin biopsies were clinically obtained from surgery for breast reduction of donors of different age (range 35–40 years old), after informed consent (kindly provided by the Plastic and Reconstructive Surgery Unit, "Salvatore Maugeri" Research and Care Institute, Pavia, Italy). Surgical biopsy (4 × 8 cm) was cut into smaller fragments (1 × 1 cm) and each of them engraved centrally with aseptic circular punch (Ø = 3 mm), removing the partial thickness of the central portion of the epidermis and dermis. The fragments were placed into Transwell inserts for 24-well multiwell plates (pore size of the membrane equal to 0.40 mm, Constar, Corning, Inc.) and treated with dressings applied in correspondence of punch. The samples were maintained in culture for different times (24, 48, 72 h) in the presence of completed DMEM medium (Dulbecco's Modified Eagle's Medium, Lonza, Milan, Italy) with 10% (v/v) foetal bovine serum (Eurobio, Courtaboeuf, F), supplemented with L-Glutamine 2 mM (Eurobio) and 100 mg/ml penicillin (Eurobio) plus 100 mg/ml streptomycin (Eurobio). The medium (900 μl/each plate) was added into the compartment below the insert in order to maintain skin viability, allowing hydration and ensuring an adequate supply of nutrients through a homogeneous diffusion from hypodermis. After different times of culture, the biopsies were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 for 8 h and processed for histological analysis. Briefly, the fragments were dehydrated through graded concentrations of ethanol and embedded in paraffin. Sections were obtained at 5–10 mm, rehydrated and stained with Harris's Hematoxylin and Eosin Y (Bio-Optica, Milan, I). Stained sections were observed with a Zeiss Axiophot microscope (Carl Zeiss, Oberkochen, G).

Table I
Mechanical properties expressed by fracture force (mN), fracture work (mN·mm) and % elongation (%E) of dressings obtained with different $CaCl_2$ concentrations (1% and 2% w/w) (mean values ± s.d; n = 3).

$CaCl_2$ concentration	TS (mN)	FW (mN·mm)	%E
1% w/w	594* (± 75)	924° (± 138)	180 (± 34)
2% w/w	1755** (± 196)	1217°° (± 69)	205 (± 22)

$p < 0.05$ * *vs***; ° *vs* °°.

2.4. Statistical analysis

Whenever possible, experimental values of the various types of measures were subjected to statistical analysis, carried out by means of the statistical package Statgraphics 5.0 (Statistical Graphics Corporation, Rockville, MD, USA). In particular, Anova one way – multiple range test was used.

3. Results and discussion

3.1. Tensile measurements

In Table I the mechanical properties, expressed as tensile strength (mN), work of fracture (mm), and % elongation of dressings obtained with different $CaCl_2$ concentrations (1% and 2% w/w) are reported. It is observed that dressings, prepared with 2% w/w $CaCl_2$, show higher values of fracture force and fracture work in comparison to dressings prepared with 1% w/w $CaCl_2$, suggesting that the highest $CaCl_2$ concentration allows for the obtainment of dressings with higher mechanical resistance. These results are in line with what recently observed by Rezvanian et al. (2017). While investigating the mechanical properties of alginate-pectin hydrocolloid films, chemically crosslinked with different concentrations of $CaCl_2$ (0.5–3% w/v), the authors observed that on increasing $CaCl_2$ concentration, the degree of crosslinking of films increased and was accompanied by an increase in the tensile strength. The improvement in mechanical properties of dressings on increasing $CaCl_2$ concentration is presumably due to the replacement of weak polymer–polymer interactions with strong interactions between calcium ions and the carboxyl groups of sodium alginate. However such interactions do not produce a significant variation in dressing elasticity, as indicated by %E values (Table I).

3.2. Hydration propensity

In Fig. 2, PBS amounts absorbed (g PBS/g dressing) by dressings after 6 and 18 h, are reported. After 6 h, 1% w/w $CaCl_2$ dressing is able to absorb a higher amount of buffer in comparison to 2% w/w $CaCl_2$ dressing, indicating a faster hydration in presence of the lowest $CaCl_2$ concentration. These results are in line with that reported in literature by other authors (Rezvanian et al., 2017). Both the dressings draw buffer inside them, starting the processes of hydration and swelling (Kianfar et al., 2014; Peles and Zilberman, 2012). Water hydrates polymer chains and enhances their mobility. In the dressings prepared with the lowest $CaCl_2$ concentration, chain relaxation occurs quickly due to the minor number of crosslinks that restrict chain relaxation due to the formation of egg box junctions (Rezvanian et al., 2017). Due to ion exchange with PBS, dressings loss their structural integrity, and ultimately dissolve. In presence of a higher crosslinking density, all these processes are slowed down (Rezvanian et al., 2017).

As demonstrated in Fig. 2, an increase in hydration time up to 18 h

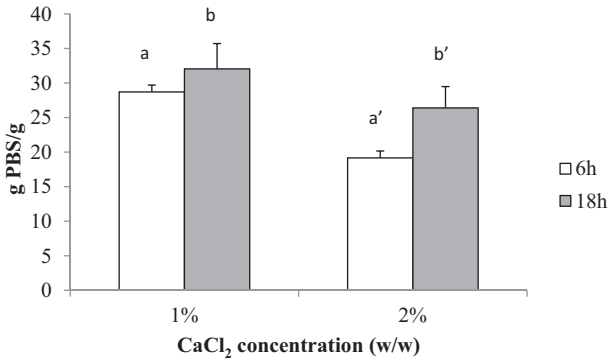


Fig. 2. Normalized PBS values (PBS amount/unit weight) observed for dressings prepared with two different $CaCl_2$ concentrations (1% and 2% w/w) after 6 h and 18 h contact with buffer (mean values ± s.d.; n = 3); ($p < 0.05$): a *vs* a'; a' *vs* b'.

does not produce an increase in the amount of buffer absorbed by 1% w/w $CaCl_2$ dressing; this is probably due to a progressive loss of dressing structural integrity and dissolution into medium. Conversely, dressing prepared with 2% w/w $CaCl_2$ shows a significant ($p < 0.05$) increase in the amount of buffer absorbed moving from 6 to 18 h hydration. The lower hydration rate that characterized $CaCl_2$ 2% w/w dressing is attributable to the higher density of cross-links due to calcium ions. No significant differences are observed between the values of buffer absorbed at 18 h by dressings prepared with the two $CaCl_2$ concentrations. Both formulations are able to absorb a considerable amount of PBS, 25-folds higher than their weight. The hydration propensity is an important characteristic of a dressing, since an excess of wound exudate can impair the regeneration process, causing tissue maceration. Moreover, exudate, in the case of chronic wounds, is rich in metalloproteinases and polymorphonuclear elastase, which are responsible for tissue destruction (Boateng et al., 2008).

3.3. Rheological measurements

The viscosity profiles of 1% w/w and 2% w/w dressings after hydration for 6 and 18 h are reported in Fig. 3. Both dressings, after hydration, yield viscous solutions with pseudoplastic behavior, characterized by a decrease in viscosity on increasing shear rate. Such behavior is functional to the lubricant/protective action required to hydrated dressings: when subjected to small stresses, the hydrated dressing is able to "absorb" the applied stress and protects the lesion area thanks to the high viscosity; on the contrary, when subjected to high stresses, the hydrated dressing offers less resistance and flows, thus acting as a lubricant (Mori et al., 2016b). 2% w/w $CaCl_2$ dressing is characterized by higher viscosity values with respect to 1% w/w $CaCl_2$.

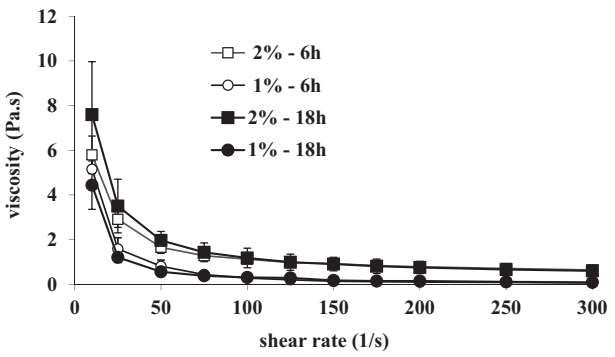


Fig. 3. Viscosity profiles of dressings prepared with different $CaCl_2$ concentrations (1 and 2% w/w) after 6 h and 18 h hydration (mean values ± s.d.; n = 3).

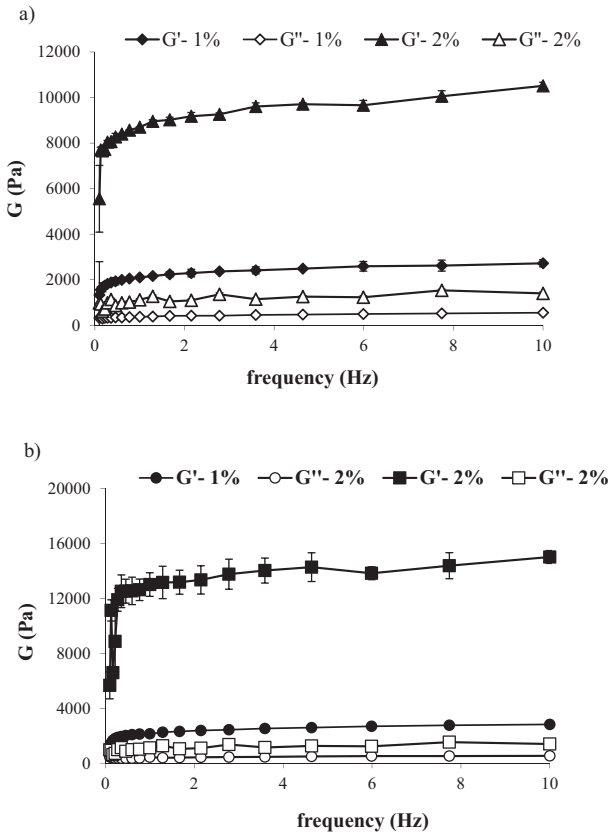


Fig. 4. G′ and G″ profiles of dressings prepared with different $CaCl_2$ concentrations (1 and 2% w/w) after 6 h (a) and 18 h (b) hydration (mean values ± s.d.; n = 3).

Even if the amount of liquid absorbed at 18 h is the same for the two dressings, the highest $CaCl_2$ concentration is responsible for a more strengthened structure, resulting in a higher viscosity profile.

In Fig. 4, storage (G′) and loss (G″) moduli obtained for both dressings after 6 (a) and 18 h (b) hydration are reported. Both dressings are characterized by greater elastic properties with respect to viscous ones, demonstrated by higher G′ than G″ profiles. This indicates that both formulations are able to recover the deformation caused by external stresses thus protecting the application area. The stronger structure of 2% $CaCl_2$ dressing is responsible for a more pronounced elastic behavior with respect to 1% $CaCl_2$ dressing.

In order to better compare the viscoelastic behavior of the two formulations at different hydration times, G′ and loss tangent values, measured at 1 Hz frequency, of the two dressings after 6 and 18 h hydration times are reported in Fig. 5. In particular, loss tangent, calculated as the ratio between G″ and G′, indicates which of the two components (elastic or viscous) prevails over the other (the smaller than 1 the loss tangent, the greater the prevalence of the elastic component on the viscous one).

As for G′ values (Fig. 5a), no significant increase is observed for 1% $CaCl_2$ dressing on increasing hydration time. This result is in line with the dressing hydration behavior: the increase in hydration time does not produce any increase in buffer absorbed (Fig. 2). On the contrary, for the 2% $CaCl_2$ dressing G′ value increases on increasing hydration time; this is in line with the slow hydration process of the dressing. The higher buffer amount absorbed is responsible for the increase in elastic properties whereas, on the basis of the dilution effect, an opposite behavior would be expected. The result obtained can be explained by the fact that the polymer network has to hydrate to a sufficient extent to display its elastic behavior. Water acts as plasticizer, allowing chain stretching and deformation recovery.

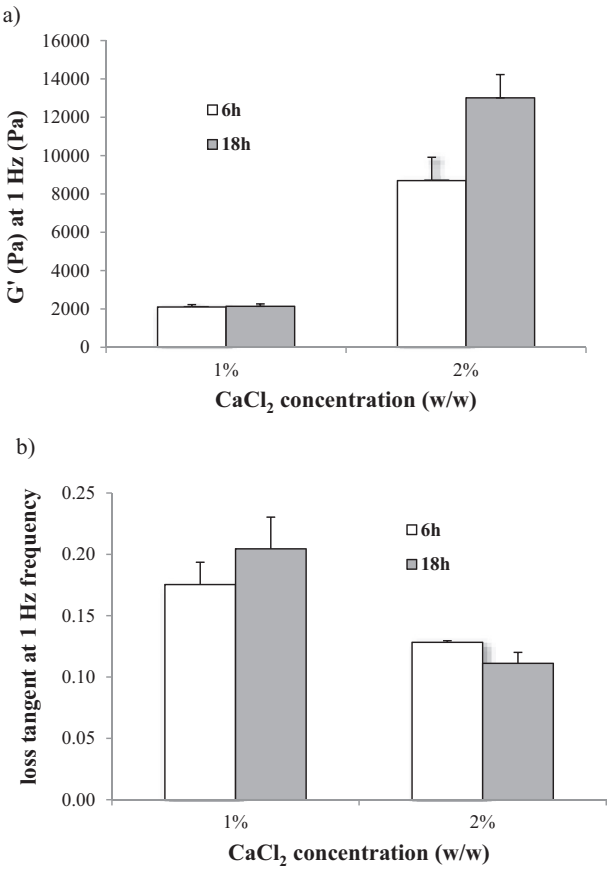


Fig. 5. G′ and loss tangent values (measured at 1 Hz frequency) of dressings prepared with different $CaCl_2$ concentrations (1 and 2% w/w) after 6 h and 18 h hydration (mean values ± s.d.; n = 3).

As for loss tangent (Fig. 5b), no significant differences are seen between loss tangent values measured at the two hydration times. In the case of the 2% w/w $CaCl_2$ dressing, this is explained by the fact that the increased liquid absorption produces an increase not only in the elastic but also in the viscous component. Since the two components, elastic and viscous, increase similarly no variation in the loss tangent value is observed. The lower loss tangent values observed for the 2% w/w $CaCl_2$ dressing with respect to 1% w/w $CaCl_2$ dressing are attributable to the stronger polymer structure due to the higher content of calcium ions.

3.4. VCM and PDGF AB release properties

Independently of $CaCl_2$ concentration, dressings showed a VCM loading capacity corresponding to the theoretical value. In Fig. 6, release profile from the dressings is reported. The different concentration of $CaCl_2$ does not affect VCM release properties, due to the fact that VCM is loaded in the alginate matrix and not in the HA particles.

In Fig. 7, the PDGF AB amount found in loaded dressings (1% and 2% w/w $CaCl_2$), in fresh PL treated with sodium citrate (as the dressings) or in PL physiological solution are reported. Sodium citrate was used to completely disrupt the particles and free the loaded amount of PDGF AB (Mori et al., 2014). PL physiological solution was used as reference.

It can be observed that PL, when treated with sodium citrate, has a lower contents in PDGF AB ($p < 0.05$) with respect to that measured in PL treated with physiological solution. This is in line with the results of a previous paper, where sodium citrate proved to produce growth factor depauperation with respect to fresh PL (Mori et al., 2014). For this reason, in order to calculate dressing loading capacity, the PDGFAB

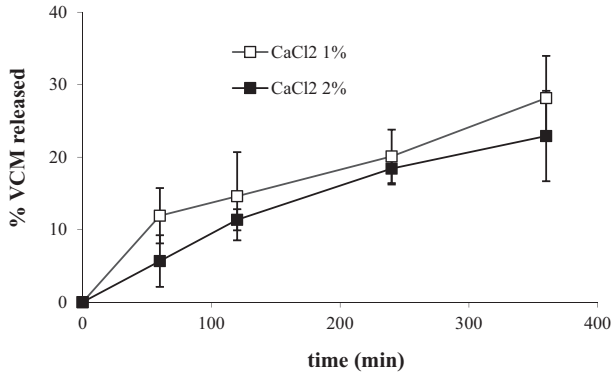


Fig. 6. % VCM released from dressings as a function of time (mean values ± s.d.; n = 3).

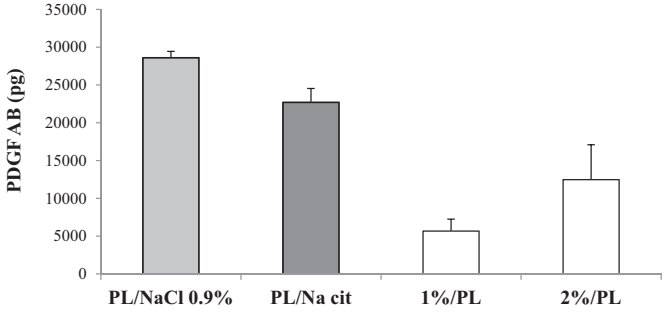


Fig. 7. PDGF AB amount (pg) loaded into dressings prepared with different $CaCl_2$ concentrations (1 and 2% w/w) and contained in PL diluted with NaCl 0.9% w/w or with 55 mM sodium citrate (pH 6.8) (mean values ± s.d.; n = 3).

amount measured into dressings (and then subjected to sodium citrate treatment) was normalized by the amount measured in PL samples subjected to the same treatment.

2% w/w $CaCl_2$ dressings load a greater amount of PDGF AB (75% of the theoretical value) with respect to 1% w/w $CaCl_2$ dressing (45% of the theoretical value). This is due to the higher degree of crosslinking occurring in presence of 2% w/w $CaCl_2$. Core-shell particles form when calcium ions diffuse through HA polymer chains and interact with ALG (Secchi et al., 2013). The capability of the PL bioactive substances contained in HA/$CaCl_2$ solution to migrate towards ALG solution depends on the barrier effect of polymeric chains at the HA/ALG interface. A higher crosslinking density is likely to impair the diffusion of high MW molecules, such as growth factors (Somo et al., 2017; Gu and Amsden, 2004).

PDGF AB release test, performed in a shaking bath, showed that both dressings are able to release PDGF AB, when in contact with a medium mimicking wound exudate (Fig. 8). Growth factor release depends on the $CaCl_2$ concentration employed in dressing preparation. A higher concentration of $CaCl_2$ is responsible for a more packed particle shell structure, which implies a lower amount of PDGF AB released. As expected, such an amount increases with time.

3.5. Cell proliferation properties

In Fig. 9a, the percent proliferation observed for fresh PL, freeze-dried PL (f.d. PL) and dressings, PL loaded and unloaded, after 72 h contact with NHDF cells is reported. Medium without serum (M w/s) is used as reference.

M w/s is characterized by a percent proliferation lower than complete medium (CM, which represents the 100%), proving the discriminating power of the test. It is observed that fresh PL and freeze-dried PL show comparable percent proliferation values, indicating that

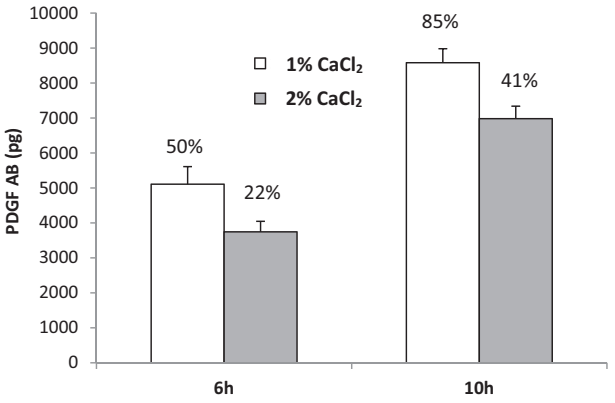


Fig. 8. PDGF AB amount (pg) released from dressings prepared with different $CaCl_2$ concentrations (1 and 2% w/w) after 6 h and 10 h (mean values ± s.d.; n = 3). Results are expressed also in percentages with respect to PDGF AB loaded into the dressings.

the freeze drying process does not impair PL activity. This is in line with the results of a previous work (Rossi et al., 2013). As expected, PL shows a percent proliferation value significantly higher ($p > 0.05$) than that observed for CM, thanks to the proliferative effect of growth factors (GFs) and bioactive substances contained in the hemoderivative.

Unloaded dressings (1% and 2% $CaCl_2$) show percent proliferation values significantly higher ($p < 0.05$) than those observed for M w/s: this is due to the presence of HA, well-known as healing promoter (Belvedere et al., 2017).

Dressings loaded with PL are characterized by % proliferation values significantly higher ($p < 0.05$) than those observed for unloaded formulations. These results indicate the formulation ability to release PL and enhance fibroblast proliferation.

In Fig. 9b, as an example, images of cell substrate after 72 h contact with 1% $CaCl_2$ unloaded and PL-loaded formulations in comparison to M w/s are reported.

3.6. Assessment of DNA synthesis by BrdU incorporation

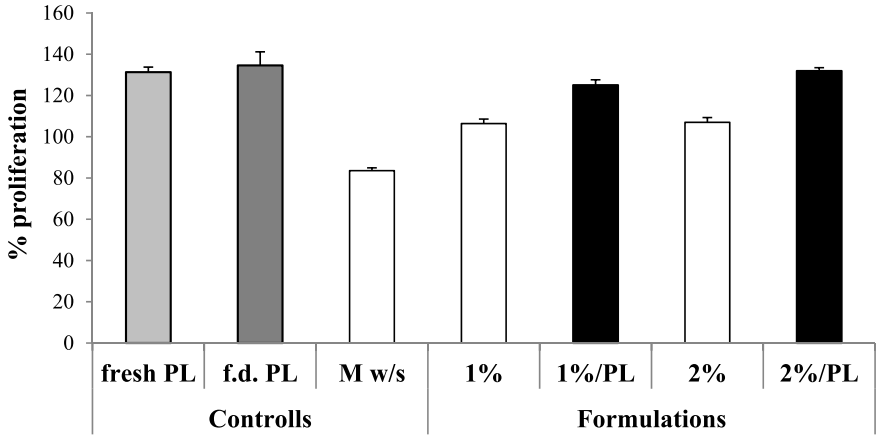
In Fig. 10, the percentage of cells in S proliferative phase, after treatment with loaded and unloaded dressings, is reported. It is observed that fresh PL is characterized by significantly higher values with respect to M w/s; this indicates the discriminant power of the test used.

Unloaded dressings are characterized by % cells in S phase higher than that of M w/s. This result is attributable to the presence of hyaluronic acid, polymer well known for its capability to promote healing (Belvedere et al., 2017). PL loaded dressings show the highest values of % cells in S phase. This proves that PL loaded dressings are able not only of increasing cell viability (Fig. 9a) but also the number of cells in proliferative phase.

3.7. Ex vivo tests on human skin biopsy

In Fig. 11 photographs of human skin strips treated for 72 h with PL loaded and unloaded dressings are shown. Skin incision with biopsy punch has determined the removal of the epidermis and dermis. After 72 h, control (untreated skin strip) still shows damage, with evident morphological alterations. Dermal matrix is markedly more organized when skin strips are treated with loaded and unloaded dressings. In such samples, collagen fibers look associated each other to form big bundles, more evident and compact in the samples treated with PL loaded dressings. In these samples, collagen bundles are oriented according to evident structural planes, suggesting an active regeneration process.

a)



b)

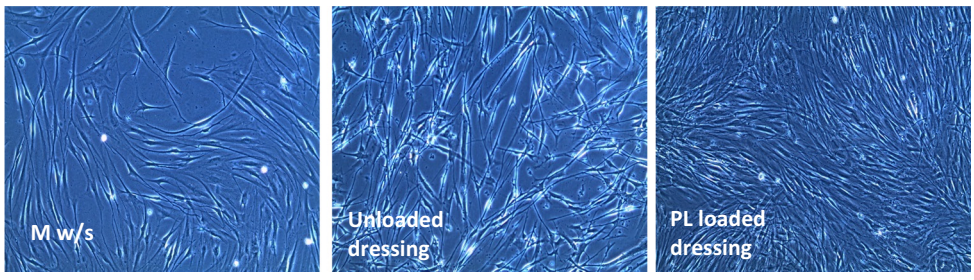


Fig. 9. Results of cell proliferation test: a) % proliferation values of dressings, loaded and unloaded with PL, prepared with different $CaCl_2$ concentrations (1 and 2% w/w), (mean values ± s.d.; n = 6). ($p < 0.05$): a *vs* c/d/f; b *vs* c/d/f; c *vs* d/e/f/g; d *vs* e/g; f *vs* g/e; b) microphotographs of the cell substrate after 72 h contact with medium without serum (M w/s), unloaded 1% $CaCl_2$ dressing and PL loaded 1% $CaCl_2$ dressing.

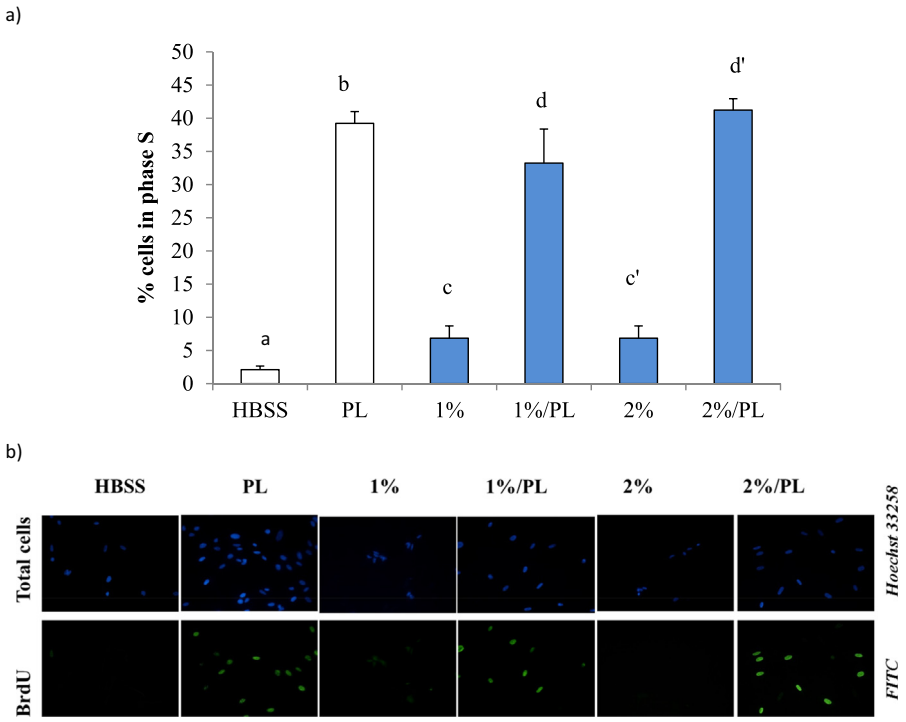


Fig. 10. Results of BrdU incorporation test: a) % cells in S phase for unloaded and PL loaded dressings (mean values ± s.d.; n = 3). b) microphotographs of proliferative cell activity: the nucleus of all cells was stained with Hoechst33258 (blue fluorescence) and the proliferative positive cells in S phase were immunostained with primary antibody anti-BrdU and FITC-conjugated secondary antibody (nuclear green fluorescence). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Conclusions

A simple method was developed for the preparation of HA particles coated with a calcium alginate shell and embedded in an alginate matrix. The dressings load the two actives considered, VCM and PL, in the external matrix and in the HA particles, respectively, thus avoiding incompatibility problems. As for PL, the highest $CaCl_2$ concentration yields the highest loading capacity, attributable to the higher degree of crosslinking. Both dressings display suitable mechanical, hydration and rheological properties. In particular, due to their sponge-like structure they absorb a higher amount of exudate with respect to alginate-based films (Ahmed and Boateng, 2018; Summa et al., 2018; Dutra et al., 2017; Rezvanian et al., 2017; Rezvanian and Amin, 2016; Mori et al., 2014; Liakos et al., 2013) and form a continuous gel layer more protective towards the lesion area with respect to the uneven layer formed by alginate-based beads (Mori et al., 2014; Liakos et al., 2013).

The use of two different $CaCl_2$ concentrations does not affect VCM release properties, but strongly influences PGFAB release from particles. The results of *in vitro* and *ex vivo* tests performed on cells and skin biopsies, respectively, support the ability of the dressings to improve healing, independently of $CaCl_2$ concentration.

The major advantages of the present formulations consist in the combined loading and delivery to skin lesion of an anti-infective drug and a pool of bioactive substances contained in PL, all together essential for the regeneration phases.

Moreover, due to the straightforward preparation technique the dressings can be easily prepared on demand in the hospital pharmacy service, where autologous hemoderivate samples are readily available as starting materials.

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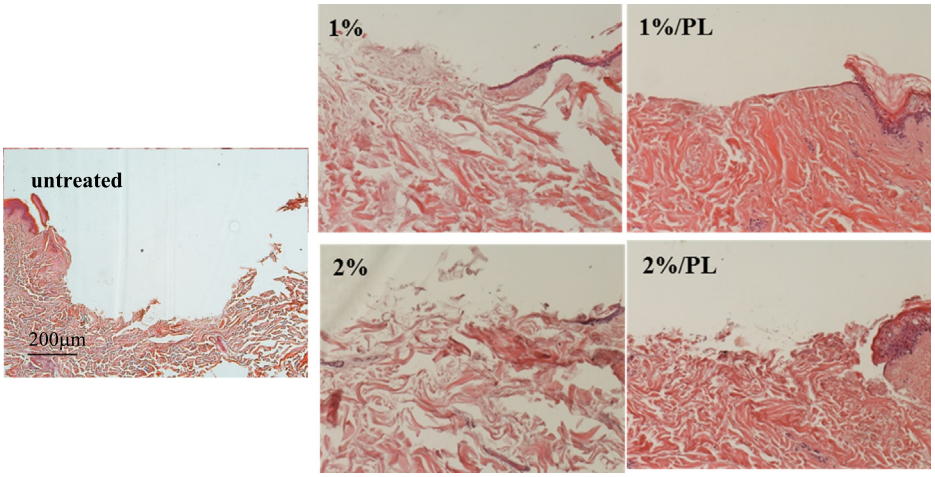

Fig. 11. Light microphotographs of hematoxylin and eosin stained sections of human skin biopsies (incised by a punch) untreated (control) or treated with unloaded (blank) or PL loaded dressings. Scale bar: 200 µm.

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