

# Analysis of a Poloxamer Hydrogel Composed of Human Mesenchymal Stromal Cells for Reepithelization of Skin Injuries

Deepika Ghai<sup>1</sup>, Mohit saini<sup>2</sup>, Sachin Kashyap<sup>3</sup>, Vipul Gupta<sup>4</sup>

<sup>1</sup>Associate Professor, Shobhit University Saharanpur

<sup>2,3,4</sup>Student, Shobhit University Saharanpur

## ABSTRACT

The body's natural response to tissue damage is wound healing. Hydrogels have compelling benefits in the healing of wounds, not only because of their permeability, biodegradability, and biocompatibility, but also because they offer an optimal environment for cell migration and proliferation. The primary goal of this work was to create and characterize a hydrogel that contained human mesenchymal stromal cells (hMSCs) in order to facilitate the healing of superficial skin injuries. hMSCs were embedded using a biocompatible biomaterial called Poloxamer 407®. The hydrogel that was created, with 20 percent (w/w) of polymer, had the best formulation in terms of its mechanical, morphological, biological, and physical characteristics.. The hydrogel's remarkable swelling capacity spoke to its ability to absorb wound exudate. The LIVE/DEAD® assay verifies that when hMSCs were injected into the hydrogels, they stayed viable for at least 48 hours. The keratinocytes' ability to proliferate and mend was unaffected by the addition of progressively higher concentrations of hMSC-loaded hydrogel to the epithelium; the entire wound healed in less than a day. Our research provides new avenues for the application of poloxamer hydrogels as skin superficial wound carriers.

**Keywords:** poloxamer hydrogels, human mesenchymal stromal cells, skin injuries.

## Introduction

The largest organ in the human body, the skin shields us from environmental harm and microbial infection. It is composed of three layers: the hypodermis, also referred to as the subcutaneous tissue, the dermis, which is the skin's inner layer, and the epidermis, which is the skin's outer layer. Merkel cells, melanocytes, and keratinocytes are found in the epidermis. Conversely, collagen, elastic fibers, glycosaminoglycans, fibroblasts, and more immune system cells make up the dermis and hypodermis (Stan et al., 2021). Traumas, severe burns, ulcers, and other injuries frequently cause damage to the skin, impairing its ability to function as a protective barrier and play a crucial part in sensory perception. In response to damage to the skin, the human body starts the healing process right away, which includes haemostasis, inflammation, proliferation, and remodelling (de Almeida et al., 2022; de Castro et al., 2023; do Nascimento et al., 2023). A wound must go through its stages in the right order for healing to occur. When the body reaches the homeostasis stage, platelets are released along with fibrin to form a thrombus or clot that seals the damaged arteries and helps stop bleeding (de Almeida et al., 2022; do Nascimento et al., 2023; Han and Ceilley, 2017). This reduces blood loss because the arteries in the

trauma area constrict. Vascular permeability rises and inflammatory cells, including neutrophils, lymphocytes, and monocytes, are activated during the inflammatory response. These cells then travel to the wound in response to chemokines. Inflammatory mediators and inflammatory cells are necessary for the clearance of necrotic tissue and foreign bodies as well as the start and control of wound healing (Huang et al., 2022). Granulated tissue with an extracellular matrix (ECM) made of freshly generated blood vessels and connective tissue forms during the proliferation stage (Liang et al., 2021). Through their migration and proliferation, keratinocytes, fibroblasts, and vascular endothelial cells create granulation tissue, neovascularization, and wound epithelial regeneration (de Almeida et al., 2022; do Nascimento et al., 2023; Huang et al., 2022). Keratinocytes play a role in the inflammatory response and have the ability to epithelialize the wounded location to seal it off (Wiegand et al., 2021). The process by which the granulation tissue created during wound healing transforms into normal connective tissue is known as remodeling (de Almeida et al., 2022; do Nascimento et al., 2023).

Wounds are sometimes divided into two categories: acute wounds and chronic wounds, depending on how long the healing stages last. Acute wounds are those that heal quickly and are typically caused by corrosive chemicals, radioactivity, mechanical trauma, heat, or electrical shock. Chronic wounds, on the other hand, are linked to certain illnesses, like diabetes mellitus, and do not follow the systematic phases and predictable timeframes that define the typical wound healing process (Naskar and Kim, 2020). According to depth, skin wounds can also be divided into three categories: full-thickness wounds, partial-thickness wounds, and superficial wounds (Da et al., 2017). Tissue engineering treatments have become more popular as possible therapeutics in recent years. Because of their potential for therapeutic benefits and regenerative ability, human mesenchymal stromal cells (hMSCs) are frequently used in the wound healing and regeneration process (Bian et al., 2022; Guillamat-Prats, 2021; Hu et al., 2018). Multipotent stromal cells, or hMSCs, are present in most tissues, but are mostly found in bone marrow and adipose tissue. They have the capacity to differentiate into mesenchymal tissue lineages, including osteoblasts, chondrocytes, myocytes, and adipocytes (Galvez-<sup>´</sup> Martın et al., 2014). Because they stimulate cell differentiation, immune modulation, growth factor secretion to encourage reepithelialization and neovascularization, and cellular infiltration, hMSCs have positive effects on skin homeostasis and lesion repair (Bian et al., 2022; Guillamat-Prats, 2021; Rodrigues et al., 2014). Poor transport and survivability of the transplanted hMSCs in the intended site of action, such as skin wounds, is the main factor limiting the therapeutic efficacy. Therefore, it is imperative that hMSCs be supplied efficiently while still in contact with the environment surrounding the skin lesion (Soriano-Ruiz et al., 2019).

Because hydrogels guarantee the stem cells' continued survival and functionality and hence increase the transplantation efficiency of the cells, they present an appealing substitute for other methods of delivering stem cells to the wound site (Kaisang et al., 2017; Stan et al., 2021). In addition to having good biodegradability, biocompatibility, adhesion, and air permeability, hydrogels are hydrophilic gels with three-dimensional elastic and porous network structures. They also effectively promote cell proliferation and migration, aiding in the healing process of wounds. Hydrogels are a great product choice for skin tissue engineering because of their qualities (Zielinska et al., 2023). Poloxamers, also known by the names of Pluronic<sup>®</sup>, Synperonic<sup>®</sup>, and Lutrol<sup>®</sup> are a family of triblock copolymers with a center block of hydrophobic polypropylene oxide (PPO) which is linked to two hydrophilic polyethylene oxide (PEO) blocks (Fakhari et al., 2017; Giuliano et al., 2020). A class of triblock copolymers known by the names Pluronic<sup>®</sup>, Synperonic<sup>®</sup>, and Lutrol<sup>®</sup>, poloxamers are connected to

two hydrophilic polyethylene oxide (PEO) blocks by a middle block made of hydrophobic polypropylene oxide (PPO) (Fakhari et al., 2017; Giuliano et al., 2020). The U.S. Food and Drug Administration (FDA) classifies poloxamer 407 as a "inactive ingredient" for various human use formulations (de Castro et al., 2023; Dumortier et al., 2006; Kaisang et al., 2017). Aqueous solutions of Poloxamer 407 exhibit thermo-reversible characteristics. Sol–gel transition temperature ( $T_{sol-gel}$ ) is a reversible property of the gelation phenomena. The poloxamer 407 aqueous solutions stay fluid below this temperature and solidify into semi-solid substance above it. The poloxamer 407 copolymer chains interface hydrophobically as a result of the thermo-gelation process. The poloxamer 407 copolymer chains combine form micellar by increasing the temperature. The hydrophobic PPO inhibits dehydrate, which causes micellization (Dumortier et al., 2006; Fakhari et al., 2017). Poloxamer 407 hydrogel's sensitivity to heat makes it easy to encapsulate cells and promotes a high number of cell attachments to the defect site (Kaisang et al., 2017). Based on all that has been said so far, the primary goal of this work was to create and characterize a hydrogel based on poloxamer and assess its suitability for the support of hMSCs intended for use in the wound healing process for superficial skin injuries.

## 2. Materials and methods

### 2.1. Materials

Pluronic® F-127 (poloxamer 407®) was purchased from BASF (Ludwigshafen, Germany). Glucose 5 %, Lactated Ringer's solution and Albumin 20 % were purchased from Grifols (Barcelona, Spain). Phosphate-buffered saline (PBS), Dulbecco's modified Eagle's medium (DMEM), collagenase type IA and Fetal Bovine Serum (FBS) were purchased from Sigma-Aldrich Chemie (St Louis, MO, USA). Penicillin–streptomycin antibiotic was purchased from Invitrogen (Merelbeke, Belgium). Trypsin was purchased from Gibco (Invitrogen, Grand Island, NY, USA). Water used in the experiments was obtained from a Milli-Q® System (Merck Millipore Co., Germany). All other chemicals were of analytical grade and used without further purification.

**2.2. Preparation** of poloxamer-based hydrogels Four formulations of hydrogels were prepared with different concentrations of Pluronic® F-127: 5, 10, 15 and 20 wt% (named HYP5, HYP10, HYP15 and HYP20 respectively). Firstly, the polymer was sterilized by autoclaving (20 min, 121 °C), and then it was mixed with a sterile packaging medium composed by 50 % of Glucose 5 %, 45 % of Lactated Ringer's solution and 5 % of Albumin 20 % at room temperature. A control formulation was prepared without Pluronic®. Hydrogels were prepared by dispersing the polymer with manual stirring and stored at 4 °C for at least 24 h to ensure complete dissolution to yield clear and homogenous hydrogels.

**2.3. Isolation** of hMSCs and cell encapsulation hMSCs were obtained from infrapatellar fat pad of patients with osteoarthritis (OA) during joint replacement surgery. Fat tissue was collected after informed consent from all patients and approval from the Ethics Committee of Clinical University Hospital of Malaga, Spain. To isolate the hMSCs, fat tissue was minced and digested using enzymatic solution of 1 mg/mL collagenase type IA at 37 °C for 1 h on a shaker. After digestion, collagenase was removed by a single wash in sterile PBS, followed by two further washes in DMEM supplemented with 10 % FBS. The cell pellet was resuspended in DMEM containing 20 % FBS, 100 U/ mL penicillin and 100 mg/mL streptomycin and cultured at 37 °C in 5 % CO<sub>2</sub>. At 80 % of confluency the cells were released with trypsin and subcultured. Cells were used between passages 3 and 5 for all the experiments.

Cells-laden hydrogels (named: HYP5-C, HYP10-C, HYP15-C and HYP20-C) were prepared in three consecutive steps. First, the poloxamer was mixed with 90 % of packaging medium to create the hydrogel as is described in Section 2.1. Second, hMSCs were suspended in the rest of packaging medium (10 %) at a cell density of  $1 \times 10^6$  cells/mL, after 24 h gelation. Finally, the cellular suspension was incorporated into a vial with the hydrogel at room temperature and under aseptic conditions. Each vial was shortly stirred to obtain a homogeneous distribution of cells and stored at 4 °C over the desired period. The composition of each hydrogel is reported in Table 1.

**2.4. Cell culture of keratinocytes** Human primary epidermal keratinocytes (HEKa) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in EpiLife medium (Gibco Waltham, MA, USA) containing HKGS (Gibco) at 37 °C in a humidified atmosphere containing 5 % CO<sub>2</sub>. HEKa cells were seeded on cell culture plates at a density of 10,000 cells/cm<sup>2</sup> and cultured until confluency when the wound-healing assay was performed.

**2.5. Biological tissue for ex vivo bioadhesion assays** Pig skin was obtained from pigs in accordance with protocols prescribed by the Animal Experimentation Ethics Committee of the University of Barcelona, Spain (CEEA-UB) code 10617. Female pigs weighing 20–25 kg, were anesthetized with intramuscular administration of ketamine HCl (3 mg/kg), xylazine (2.5 mg/kg) and midazolam (0.17 mg/kg). Propofol (3 mg/kg) was administered via atrial vein after sedation and immediately afterwards they were intubated and maintained under anaesthesia inhaled with isoflurane. Finally, three animals were sacrificed under veterinary supervision at the Animal Facility of the Bellvitge Campus of the University of Barcelona (Spain) using an overdose of sodium thiopental. Pig skin was maintained in HBSS and refrigerated until experiments (Amores et al., 2014). Before use, the pig skin was superficially cleaned with gauze soaked in 0.05 % dodecyl sulphate solution, followed by distilled water.

**2.6. Macroscopic analysis of the prepared hydrogel formulations** The physical appearance of the prepared cells-free hydrogels and cells-laden hydrogels was inspected visually after 72 h of preparation in relation to macroscopic aspect and color.

**2.7. Cell viability Viability of hMSCs** embedded in the hydrogel formulations was determined by trypan blue dye exclusion staining and posterior cell counting in a Neubauer chamber. It was performed at 0, 24, 48 h after embedding cells into hydrogel at 4 °C and 37 °C. A fluorescence microscope (Olympus IX71; Olympus, Tokyo, Japan) was used to observe the cells. Each sample was counted three times and the average was calculated. The percentage of viability was calculated using the following equation:  $Cellviability(\%) = \frac{Numberofviablecells}{Totalnumberofcells} \times 100$  (1)

## 2.8. Cell distribution

The hMSCs distribution in each hydrogel was evaluated by Live/ Dead kit assay (Invitrogen Inc, USA), following the manufacturer's instruction. It was performed at 0, 24, 48 and 72 h after embedding cells into hydrogel at 4 °C and 37 °C. Briefly, cells-laden hydrogels were stained with 5 µL of 2 µM calcein AM and 10 µL of 4 µM ethidium homodimer-3 in 4 mL of sterile PBS (with calcium), and incubated in the dark for 30 min, to stain dead cells in red and live cells in green. After staining, samples were washed with PBS and observed under a confocal microscope (Zeiss LSM 710). Images were evaluated

with the software ImageJ. For each sample, four regions were counted to obtain an average value of percent viable cells.

### 2.9. hMSCs karyotyping analysis

The hMSCs were analyzed before and after embedding cells into hydrogel. Karyotype analysis was performed by G band techniques (Bayani and Squire, 2004). In order to obtain chromosomal preparations, the hMSCs were treated with 0.8 mg/mL colchicine and incubated at 37 °C for 1.15 h. Briefly, cells were washed with 1 mL trypsin twice. Then, 2 mL trypsin was added at 37 °C for 2 min and centrifuged at 400g for 10 min with 1 mL FBS. The pellet was suspended in 5 mL 75 mM KCl at 37 °C for 20 min and centrifuged at 400 g for 10 min. The cells were fixed with 5 mL methyl alcohol–acetic acid mixture (3:1 v/v) and centrifuged at 400 g for 10 min, this process was repeated twice more. For each karyotype 20 metaphases were analyzed. The result was described to account the recommendations from the International System for Human Cytogenetic Nomenclature (Gonzalez Garcia and Meza-Espinoza, 2006).

### 2.10. Physicochemical characterization of hydrogels

**2.10.1. Gelation time and osmolality** For the determination of gelation time, 5 mL sample was put into a transparent vial with a magnetic bar and placed in a low temperature water bath. The solution was then heated from 10 to 37 °C ± 0.1 °C while being stirred (200 rpm). The gelation time and gelation temperature was measured in triplicate, once the magnetic bar stopped moving due to gelation (Soriano-Ruiz et al., 2020). The gelation process was also studied using the test tube inversion test (Dong et al., 2023). The states of the hydrogels at different temperatures were photographed. Osmolality was determined using a micro-osmometer model 3320 (Advanced Instruments, Inc., Norwood, MA, USA). This instrument measures the freezing point depression (FPD), which is directly proportional to the concentration of osmotically active compounds in aqueous solutions.

**2.10.2. Swelling test** The swelling ratio (SR) was assessed by a gravimetric method (El Moussaoui et al., 2021). The swelling properties of hydrogels were determined using lyophilized samples. Lyophilized dry hydrogels samples (0.05 g, n = 3) were incubated in 100 µL PBS at different pH (pH = 7.4 and pH = 5.5) at 37 °C. At selected time intervals the swollen hydrogels were removed and weighed after blotting the excess of PBS with a filter paper. Hydrogels were then replenished with fresh PBS. The swelling ratio was calculated based on the following:

$$SR (\%) = \frac{W_s - W_d}{W_d} \times 100$$

Where  $W_s$  and  $W_d$  are the weight of the swollen and dried hydrogel samples respectively.

**2.10.3. In vitro degradation analysis** the degradation as percentage of weight loss (WL) was calculated by incubating of fresh hydrogels ( $W_i$ ) (0.5 g) in 1 mL PBS at different pH (pH = 5.5 and pH = 7.4) and at 37 °C. At regular time intervals, hydrogels were dried and weighed ( $W_t$ ) after blotting the surface PBS. Briefly, the samples were put back in fresh PBS. The degradation was determined in triplicate at each time point and for each pH (El Moussaoui et al., 2021). WL was calculated as follows:

$$WL(\%) = (W_i - W_t) / W_i \times 100$$

**2.10.4. Spreadability test** Spreadability was determined using a manual technique. After 24 h encapsulation, a cylindrical shaped of hydrogel (0.5 g) was obtained using a microtome. Then, the hydrogel was pressed between the surface of microtome and a graduated glass plate (6 × 6 cm, 25 g), on which weights of 20, 50, 100, 200 and 500 g were place at intervals of 1 min. The perpendicular diameters were determined during each interval and the results were expressed as the area (mm<sup>2</sup>) (Contreras and Sanchez, 2002). The spreadability of cells-laden hydrogel was determined in triplicate at 25 °C and 37 °C and compared with a free-cells hydrogel.

**2.10.5. Rheological analysis** The rheological characterization was conducted using the same equipment, under both oscillation and rotational modes, to evaluate changes induced by the addition of cells. Investigations into the properties of hydrogels were undertaken, with a particular focus on attributes such as viscosity, flow properties, and viscoelasticity. It is critical to understand these rheological properties in the process of designing and selecting hydrogels for specific applications. This ensures that their behavior under different conditions aligns with the intended purpose.

**2.10.5.1. Oscillatory studies.** The influence of temperature on the rheological properties of the formulations was examined through a temperature sweep ranging from 10 to 40 °C. This operation was conducted using a Haake RheoStress 1 rheometer (Thermo Fischer Scientific, Karlsruhe, Germany) connected to a Thermo Haake Phoenix II + Haake C25P temperature controller. The rheometer was outfitted with a mobile Haake PP60Ti plate (60 mm in diameter), operating in oscillation mode at a constant stress of 0.5 Pa. This value was selected based on a prior stress sweep test, which demonstrated that this stress level fell within the viscoelastic region. The rheometer operated at a standard frequency of 1 Hz, a value selected after a frequency sweep test. Each sample was equilibrated by placing it within the plate-plate sensor system (with a gap of 1 mm) for a duration of 5 min, enabling it to reach the initial temperature of 10 °C. Subsequently, the temperature was incrementally increased from 10 to 40 °C, adhering to a controlled ramp speed sustained over a duration of 2070 s. Throughout this process, measurements were made of the elastic modulus ( $G'$ ), the viscous modulus ( $G''$ ), and the complex viscosity ( $\eta^*$ ). Determining the crossover of moduli ( $G' = G''$ ) is a commonly employed method for identifying the sol–gel transition point in thermosensitive hydrogels [30,31].

**2.10.5.2. Rotational studies.** Flow behavior and viscosity determination were carried out with the same rheometer, but equipped with a Haake C60/2Ti mobile cone (60 mm diameter, 0.102 mm gap, and 2° angle) operating in rotation mode. Each sample was equilibrated by placing it between the cone-plate sensor system (0.102 mm gap) for 5 min to attain the running temperature. Viscosity curves and flow curves were recorded for 1 min during the ramp-up period from 1 to 100 s<sup>-1</sup>, 1 min at 100 s<sup>-1</sup> (constant shear rate period), and finally during the ramp-down period from 100 to 1 s<sup>-1</sup>. Data from the flow curve (when they were found to be non-Newtonian) were fitted to different mathematical models, equations (4) – (8) using the Prism®, V. 3.00 software (GraphPad Software Inc., San Diego, CA, USA) in order to determine the flow type

$$\tau = \tau_0 + \eta_p \times \dot{\gamma} \quad \text{Bingham} \quad (4)$$

$$\tau = k \times (\dot{\gamma})^n \quad \text{Ostwald – De – Waele} \quad (5)$$

$$\tau = \tau_0 + k_1 \times (\dot{\gamma})^n \quad \text{Herschel – Bulkley} \quad (6)$$

$$\sqrt{\tau} = \sqrt{\tau_0} + \sqrt{k_1 \times \dot{\gamma}} \quad \text{Casson} \quad (7)$$

$$\frac{\eta - \eta_\infty}{\eta_0 - \eta_\infty} = \frac{1}{(1 + (\lambda\dot{\gamma})^m)} \quad \text{Cross} \quad (8)$$

The mean viscosity value (Pa•s) was determined from the constant shear section at  $100 \text{ s}^{-1}$  of the viscosity curve with respect to temperature. The determination of the disturbance of the microstructure during the test evaluated by determining the area of hysteresis loop

**2.10.6. Porosity analysis** The porosity of the hydrogel was calculated according to the Archimede's principle (El Moussaoui et al., 2021). The porosity analysis was carried out by immersion of the lyophilized hydrogels (0.1 g) in 200 mL PBS (pH = 5.5 and pH = 7.4) at room temperature. The submerged mass of the hydrogel was recorded and then the hydrogel was removed, and the wet mass was recorded. Triplicate pH = 5.5 and pH = 7.4 samples were used for the study. Porosity of hydrogels were calculated using the following equation:

$$\text{Porosity (\%)} = \frac{M_w - M_D}{M_w - M_{SUB}} \times 100 \quad (9)$$

where  $M_w$  is the water saturated wet mass of the hydrogel,  $M_D$  is the dry mass of the hydrogel, and  $M_{SUB}$  is the submerged mass of the hydrogel

**2.10.7. Water contact angle measurement** The surface wettability of the hydrogels was determined by calculating the water contact angles in fresh samples at room temperature. Contact angles were determined using Contact Angle Goniometer (Raméhart Instrument co, USA). A uniform drop of distilled water (30  $\mu\text{L}$ ) was dispensed on the hydrogel surface. The contact angle was evaluated for a precise interval of time from 1 to 10 min until the drop had disappeared. Each measurement was carried out in triplicate and the average value was reported.

**2.10.8. Conductivity** Electrical conductivity of the hydrogels was measured with a conductivity meter Crison EC-Meter BASIC 30+ (Crison Instruments, Alella, Spain). The hydrogels were dilute in in PBS 1:20 (pH = 5.5 and pH = 7.4). An electrode of the device was dipped into a glass vial of the samples. Data were expressed as mean  $\pm$  SD (n = 3).

**2.10.9. Determination of the zeta potential** Surface electrical properties of the hydrogels were determined in a Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK), which estimates the zeta potential of samples placed in cells (cuvettes), determining the electrophoretic mobility through Henry equation by measuring the velocity of the particles using Laser Doppler Velocimetry (ZetaSizer, 2023). The zeta potential of the hydrogels was measured at different pH values (pH 4–pH 8), and under a constant  $10^{-3}$  M  $\text{KNO}_3$  concentration after suitable dilution (0.1 % w/v) of the hydrogel. Zeta potential determinations were determined after contact during 24 h under mechanical stirring (50 rpm) and at room temperature (Pretel et al., 2017). Values are reported as the mean  $\pm$  SD of three replicates.

#### **2.10.10. Leakage test**

The leakage test was conducted in agar gel surface (Esposito et al., 2016). For this task agar (1.5 %, W/W) was dissolved in simulated PBS 7.4 and PBS 5.5 and stirred at  $95^\circ\text{C}$  until solubilization. Enough quantity was added to form a thin layer in glass slides and kept at room temperature until the gel was obtained. Furthermore, assayed samples were colored with methylene blue to improve observation. About 50 mg of samples were placed onto the end of agar slides, which were put vertically at an angle of  $90^\circ$  and maintained at  $4, 25$  and  $37 \pm 0.5^\circ\text{C}$ . The running distance covered by samples was measured 1, 5 and 10 min after the placement. Leakage value was calculated as the mean  $\pm$  SD of three replicates.

### 2.10.11. Evaluation of bioadhesive properties

The *ex vivo* bioadhesive properties of the formulation are measured by testing its resistance or tension when attempting to break the bond between the tissue epithelial membrane and the formulation. To do this, we developed a simple device for determining the bioadhesive force of the formulation (Li et al., 2012; Mei et al., 2017; Yang et al., 2017). We cut pieces of pig skin to a size of 2.5 × 2.5 cm and fixed them onto two plates, respectively. One plate was fixed onto a stainless-steel base, and the other was connected to a firm thread that fastened a light plastic beaker through a fixed little crown block. We placed 0.3 g of the sample between two pieces of tissue and slightly pressed the upper plank using our hand. We then dropped water into the beaker at a speed of 1.0 mL/ min until the two planks were pulled apart due to the gravity of the water on the beaker. The test was carried out at different temperatures, 32 and 37 °C. Finally, we weighed the beaker with water and calculated the bioadhesive force (F, mN/cm<sup>2</sup>) using the following equation:

$$F = \frac{W \times g}{A} \quad (10)$$

where W is the mass of water, g is the acceleration due to gravity, and A is the surface area of the applied formulation. The bioadhesive force was presented as mean values ± SD of three replicates. The bioadhesive property of poloxamer 407 formulations was also evaluated measure the influence of the samples on the zeta potential of mucin (Abdellatif et al., 2020; Mendes et al., 2018). Each sample was dispersed with an equivalent amount of 1 % (w/v) of e mucin solution under stirring. The mixtures of hydrogel-mucin were allowed to equilibrate overnight at room temperature. The surface charge of samples was detected using a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK). Readings were reported as the mean ± SD of nine replicates.

### 2.10.12. Extrudability study

This test measures the required force to remove the hydrogel from the container. The extrudability (E, g/cm<sup>2</sup>) of the development hydrogels was evaluated by measurement of the weight necessary to be applied to remove an amount of the hydrogels from 2, 5 and 10 mL syringes. About 2, 5 and 10 g of each formulation were carefully loaded into syringe avoiding the formation of air bubbles. The device was vertically placed on a support and known weight was added to its plunge. The plunger was pressed down and the extruded weight was recorded. The system was maintained at 4 and 25 ± 0.50 °C, to simulate application conditions. The extrudability of developed hydrogels was calculated according to the following equation:

$$E = \frac{W}{A} \quad (11)$$

where W is the weight applied (g) to extrude the gel from de tube and A the area (cm<sup>2</sup>) of the extruded hydrogel from the tube. The data obtained were expressed as the mean ± SD of three replicates.

### 2.10.13. Spray angle

To measure the spray angle, a plastic with a dip tube diameter of 1.2 mm and an aperture size of 0.3 mm was used. The spray was actuated in horizontal direction onto a white paper mounted at a distance of 15 cm from the nozzle. Assayed samples were colored with methylene blue to improve observation. The test was carried out at 4, 10 °C and 25 °C to simulate application conditions. The radius of the circle,



formed on the paper, was recorded (Umar et al., 2020). Spray angle is calculated from the following equation:

$Spray\ angle = \tan^{-1}(l/r)$  (12) where  $l$  is a distance of the paper from the nozzle and  $r$  is the radius of the circle.

**2.10.14. Thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC)** Thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC) were performed with a Mettler Toledo mod. TGA/DSC1 calorimeter (Mettler Toledo, Barcelona, Spain), equipped with a sensor and FRS5 microbalance (precision 0.1  $\mu$ g) Samples (11–24 mg) have been sealed in aluminium-cells and brought to the initial temperature. The heating rate was 10  $^{\circ}$ C/min in the 28–950  $^{\circ}$ C temperature range. Nitrogen was used as purge gas under 50 mL/min flow. The DSC thermo gram has also been recorded in the temperature range from  $\approx$  5 to 70  $^{\circ}$ C. The measures were performed using a METTLER-TOLEDO mod. TGA / DSC1 instrument. Samples (17 mg) were sealed in aluminium-cell and brought to the initial temperature. The heating rate was 2  $^{\circ}$ C/min, and nitrogen was used as purge gas under 50 mL/min flow.

## 2.11. Morphological characterization

### 2.11.1. Size

Mean particle diameter and polydispersity index (PdI) were analyzed by photon correlation spectroscopy (PCS, Zetasizer Nano ZS, Malvern Instruments Ltd., UK) at 4, 32 and 37  $^{\circ}$ C after suitable dilution of the hydrogel (0.1 %, w/v). Each measurement was carried out in triplicate and the average value was reported.

**2.11.2. Environmental scanning electron microscopy analysis** The internal microstructures of hydrogels were analyzed using a scanning electron microscopy (SEM). Sample preparation was performed by freezing in liquid nitrogen and freeze-drying. Then, it was sputter coated with a thin layer of silver. The prepared samples were observed using a Field Emission Scanning Electron Microscope System (FE-SEM; Gemini Carl ZEISS). Lyophilization induces rapid cooling and phase separation followed by sublimation under vacuum, which alters the porosity of hydrogels and leads to void formation in their structure (Soriano-Ruiz et al., 2019a). Therefore, we also analyzed the samples using the critical point drying technique. HYP20 and HYP20-C hydrogels were fixed with a solution of glutaraldehyde 2.5 % w/v (Merck, Darmstadt, Germany) in 0.1 M sodium cacodylate buffer (Electron Microscopy Sciences, Hatfield, PA, USA), during 2 h at 4  $^{\circ}$ C, and then washed three times every 15 min with the same buffer solution. Hydrogels were processed using the critical point drying technique. First, they were fixed with 1 % w/v osmium tetroxide for 1 h at room temperature and washed with bidistilled water three times every 5 min. Then, they were dehydrated in a series of ethanol solutions with increasing concentration (50 %, 70 %, 90 %, and 100 %) at 4  $^{\circ}$ C for 15 min each. The samples were critical point dried in a Leica EM CPD300 dryer (Leica Microsystems, Wetzlar, Germany). Finally, samples were deposited on a double-coated carbon conductive tape, and covered by evaporating them in a high-vacuum thermal evaporator Emitech K975X before being observed in high-vacuum mode.

**2.11.3. Lyophilization and relationship with the extrusion process** 1.0 mL of each formulation (HYP20 y HYP20-C) was loaded into a syringe at 4  $^{\circ}$ C and 25  $^{\circ}$ C. The hydrogels were unloaded into

separate Eppendorf tubes once (1X) with a syringe. The samples were immediately lyophilized, fractured cryogenically, metallized and then analyzed through a microscope. For scanning microscope analysis, samples were mounted on SEM holders using a double carbon tape and also were covered with a gold thin film in order to improve their electrical conductivity (Bio-Rac SC-510). The samples were observed with a Jeol JSM-7001F (Jeol, Japan) operated at 15 kV performed on the TEM-SEM Electron Microscopy Unit from Scientific and Technological Centers (CCiTUB), Universitat de Barcelona.

### 2.12. Wound-healing assay

The day of the assay the medium was changed and HEK a confluent cultures were scratched with a 200  $\mu$ L pipette tip in the middle of the well and HYP20-C hydrogel were added at different proportions (30,000, 60,000 and 120,000 hMSCs/well). For better visualizing the HEK a layer, the highest concentration was also tested by separating the hMSCs cells by introducing them into an insert. Before and after 4 h incubation, the width of wounds was photographed with a fluorescent cell imager (Zoe, Bio-Rad, Germany). The wound area was calculated using the Image J public domain software. The percentage of wound area reduction was calculated from the following equation:

$$\text{Woundclosure}(\%) = \left( \frac{A_0 - A_t}{A_0} \right) \times 100 \quad (13)$$

where  $A_0$  is the area of the wound (cell-free area) measured at the initial time, and  $A_t$  is the area of the wound measured 4 h after scratching (Augustine et al., 2018). 2.13. *Statistical analysis* Tests for significant differences between means were performed by Student's *t*-test or one-way ANOVA using the Prism®, V. 3.00 software (GraphPad Software Inc., San Diego, CA, USA). Differences where *p* less than 0.05 were considered statistically significant. Experiments were repeated on three different samples and the results were expressed as mean  $\pm$  standard deviation (SD).

## 3. Results 3.1. Effect of Pluronic-127 concentration on hMSCs

### Conclusion

hydrogel loaded with hMSCs was developed for its use in the treatment of skin superficial wound. From a physicochemical perspective, HYP20-C had an optimal swelling property, degradation rate, porosity, surface properties, spreadability and extrudability. It also exhibited the required rheological properties necessary for its use as in situ gel systems for topical administration. Furthermore, hydrogel showed hydrophilic properties that play a crucial role in preventing bacterial infections, promoting cell migration, and preventing the loss of body fluids and essential nutrients *in vivo*. The HYP20-C provided a good environment for cell viability. In addition, the wound healing assay indicated that the HYP20-C did not cause any damage to the cell layer and showed complete restoration of keratinocyte presence at the wound site in a shorter period of time. Based on all results discussed above we demonstrated that HYP20-C is a suitable formulation for its use in tissue engineering and can be proposed as a potential candidate for superficial wound healing.

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