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Additional Information

A new waterborne chitosan-based polyurethane hydrogel as a vehicle to transplant bone marrow mesenchymal cells improved wound healing of ulcers in a diabetic rat model

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12

13 Abstract

14 Foot ulcers, a common complication of diabetes, can cause physical incapacity and are

15 derived from several factors, including poor wound healing. New therapeutic strategies are

16 needed to minimize this complication for the sake of patients' health. We therefore developed

17 a new chitosan- polyurethane hydrogel membrane (HPUC) and the test results confirmed

18 that HPUC present low cytotoxicity and improved wound healing when used with

19 mononuclear bone marrow fraction cells in the diabetic rat model. The biodegradable

20 hydrogels were produced in block copolymer networks with a combination of chitosan blocks

21 and biodegradable polyurethane. The membranes were characterized by FTIR, ^{13}C-NMR

22 and thermogravimetry. Swelling and hydrolytic degradation were also evaluated. The non-

23 solubility of the membranes in good solvents and the chemical characterization confirmed

24 that the network structure was formed between the PU and the chitosan through

25 urea/urethane bonds. The findings confirm that the HPUC have interesting properties that

26 make them suitable for wound healing applications.

28

1. Introduction

The diabetes mellitus (DM) group of metabolic diseases, whose main characteristic is their hyperglycemic status, can be caused by the lack of insulin, a deficit in the production or reception of this hormone, among other causes. This disease is associated with several complications such as nephropathies, retinopathies, cardiovascular diseases, ulcers and limb amputation. It is a major public health problem worldwide due to its high prevalence and morbidity (Tao, Shi, & Zhao, 2015). Recurrent infections are caused by reduced immunity, a phagocytic function associated with hyperglycemia and poor vascularization (Baltzis, Eleftheriadou, & Veves, 2014; Boulton, 2013; Dinh et al., 2012; Hilfiker, Kasper, Hass, & Haverich, 2011). Cell therapy with bone marrow mesenchymal stem cells has proved to be an effective tissue repair strategy. Although the pathways involved in this repair process have not been fully identified, it is known that transplanted cells release growth factors that modulate the immune response, organize tissues, promote angiogenesis and stimulate cell differentiation. Tissue engineering can aid in the regenerative process by developing new biomaterials that can regenerate wounds, prevent contractions and protect against infectious agents. The tissue repair process occurs in three distinct phases: inflammation, formation and remodeling of new tissue (Baltzis et al., 2014; Boulton, 2013; Dinh et al., 2012; Hilfiker et al., 2011). Besides mitigating the inflammatory process, effective wound healing dressings must have the following characteristics: wound adherence, histocompatibility, fluid loss control and sterility.

In tissue engineering applications, biopolymers are of great interest in the manufacture of three-dimensional supports for cell culture and transplant. Chitosan (CHS) has been shown to be a highly suitable biopolymer for these applications (Ching Ting Tsao et al., 2011; Hilmi et al., 2013; T. Jiang et al., 2010; Moise et al., 2012). In addition to its good biocompatibility and non-toxicity, CHS has properties of particular interest as a wound-healing dressing, such as antimicrobial activity and excellent hemostatis (Ouyang et al., 2018). However one of its limiting factors is that it is not soluble in most organic solvents, although it can be dissolved in diluted acid solutions, and this hinders its use in the manufacture of complex structures. Chitosan has reactive amine and hydroxyl side groups occurring in the polymer chains, which can be modified by derivatization (Andrade et al., 2011; Casettari et al., 2012; Merchant et al., 2014). Alkaline treatment has also been used to reduce its molecular weight, if required (Barikani, Honarkar, & Barikani, 2010). These characteristics make it versatile to being

modified by grafting specific side groups or complexing with other classes of polymers. Polyurethanes (PUs) synthesized in aqueous dispersion have recently attracted much attention due to their physicochemical properties and biocompatibility (X. Jiang et al., 2007; Rowlands, Lim, Martin, & Cooper-White, 2007; Wang, Ping, Chen, & Jing, 2006; Zuber, Zia, Mahboob, Hassan, & Bhatti, 2010). The polyester poly (DL lactide-co-glycolide) (PLGA) is the most frequently used polyester in the synthesis of PU-based biomaterials. It has been approved by the FDA for different applications and has been used for many years in the development of medical devices as well as in raw materials for the pharmaceutical industry. Another of its benefits is that its hydrolysis products can be uptaken in the cellular metabolic pathway (Jovanovic et al., 2010; Page et al., 2012).

Bioresorption of CHS takes place by the enzymatic degradation of the polymer chains, although this is a slow process and depends on the location of the implant. Our hypothesis is that combining chitosan in a block copolymer network with other polymers susceptible to hydrolytic degradation will allow fast degradation of the material in aqueous media while maintaining the hydrogel behavior characteristic of chitosan and its proven regeneration capacities. This hypothesis is based on previous works on polymer blends or block copolymers containing chitosan as one of the components (Ünlü, Pollet, & Avérous, 2018, Gámiz-González, Vidaurre, & Gómez Ribelles, 2017; Hu et al., 2012; Dunia M. García Cruz, Gomez Ribelles, & Salmerón Sánchez, 2008). The novelty of this work was synthesizing PU-chitosan hydrogel membrane (HPUC) by introducing the PLGA blocks into soft PU segments. The hydrogel was formed in two steps, with the reaction of PU prepolymer via aqueous dispersion with unmodified chitosan. In this way, we produced a hydrogel for wound healing by combining CHS and PLGA blocks in a block copolymer network. Thus, we took advantage of PLGA's hydrolysis properties to control degradation. After the physical and chemical characterization of the hydrogel membrane, a biocompatibility test was performed with bone marrow mononuclear cells (BMMNC) before evaluating the membrane combination as a wound-healing dressing and for BMMNC injections in a rat diabetic model. The promising results provided evidence that HPUC plus BMMNC may be used to enhance the wound healing process.

2. Experimental

2.1. Materials

92 Chitosan [poly-(a-1/4)-2-amino-2-deoxy-D-glucopyranose] was purchased from Sigma-
93 Aldrich with viscosity-average molecular weight of 1500 kDa determined by Mark-Houwink
94 equation using 0.1M CH_3COOH/0.2M NaCl as a solvent system at 25ºC and average
95 deacetylation degree (DD) of 75% determined by first derivative UV spectrophotometry
96 (Dunia Mercedes García Cruz, Salmerón-Sánchez, & Gómez-Ribelles, 2012).
97 Hexametylene diisocyanatee (HDI), 2,2- bis(hydroxymethyl) propionic acid (DMPA),
98 Triethylamine (TEA), Tin(II) 2-ethylhexanoate, (3S)-cis-3,6-Dimethyl-1,4-dioxane-2,5-dione
99 (L-Lactide), 1,4-Dioxane-2,5-dione (glycolide) and Streptozocinwere purchased from Sigma-
100 Aldrich. All materials were used as received without further purification.

2.2. Membranes preparation

102 The hydrogel based on waterborne polyurethane-chitosan (HPUC) was obtained from a
103 polyurethane prepolymer and chitosan. The PU prepolymer was obtained from a polyester
104 diol, previously synthetized, HDI and DMPA. Chitosan was used as received.

2.2.1. Synthesis of the polyester diol

106 The poly(lactid-co-glycolide)diol (PLGAdiol) was synthesized according to Ivirico et al. (2009)
107 (Escobar Ivirico, Salmerón-Sánchez, Gómez Ribelles, & Monleón Pradas, 2009), with some
108 modifications for obtaining a polyester copolymer with Mn of 2000 Da, from a mixture of 50:50
109 of lactid acid and glycolide. Briefly, for this purpose 0.06 mol of lactide and 0.06 mol of
110 glycolide were reacted with 0.01 mol of ethylene glycol at 120ºC for 4 hours at 23 mbar. After
111 synthesis, the polymer was dissolved in acetone, precipitated in hexane under stirring and
112 dried in a vacuum.

2.2.2. Synthesis of the PU prepolymer

114 All PU prepolymers were obtained at a ratio 2/1/1 of NCO groups in HDI / OH groups in
115 PLGAdiol / OH groups in DMPA with 7% (wt%) of the internal emulsifier DMPA in relation to
116 the polyester diol. The internal emulsifier was 100% neutralized with TEA. The amounts of
117 PLGAdiol for each HPUC series are listed in Table 1. The reaction was carried out in a three-
118 necked round bottom flask using the adequate amounts of the polyester diol PLGA, HDI,
119 DMPA and Tin(II) 2-thylhexanoate in 5 mL of dimethyl sulfoxide. The reaction proceeded at
120 70 °C for 2h under N_2 atmosphere and magnetic stirring. TEA was then added to neutralize -
121 COOH groups of DMPA, and the reaction medium stirred for 30 min.

122

2.2.3. Synthesis of hydrogel based waterborne polyurethane-chitosan (HPUC).

Chitosan (0.01 g/mL) was solubilized in a 1% acetic acid solution for 45 min at 40 °C in an ultrasonic bath. Before use, the Chitosan solution was filtered (45 μm filter) and transferred to an addition funnel. The Chitosan solution was added dropwise to the PU prepolymer solution under stirring at 70 °C. After homogenization, the final solution was cast in a poly(tetrafluor ethylene) Petri dish and kept in an air circulating oven at 60 °C for 24h to allow the cross-linking reaction and solvent evaporation. The membranes thus formed were washed by immersion in deionized water for three days, changing the water every day. The membranes were finally washed with acetone in a refluxing apparatus for 4 h and then dried in a vacuum at room temperature.

2.3. Characterization of HPUC membranes

Infrared spectroscopy was performed on a Perkin-Elmer Spectrum 100 FT-IR (ATR) spectrometer. Solid- state ^{13}C NMR was performed on an Agilent DD2 500MHz (11.7T) NMR spectrometer. Thermogravimetric analyses were conducted on a Mettler-Toledo TGA/SDTA851e –FL1600. The samples were heated from 30°C to 900°C at a 10 °C/min heating rate in an N_2 atmosphere (50ml/min).

2.4. Solubility test and water uptake

The dry samples were cut into pieces weighing around 50 mg. For solubility tests, the samples were immersed in each solvent in a vial and shaken at room temperature. The water uptake of the samples after being immersed for different times was evaluated as:

$$S= [(M_f- M_i/)M_i]* 100, \text{ where:}$$

M_i is the weight of the dry sample at time t = 0 s and M_f is the weight of the wet sample at immersion time t.

2.5. Degradation test

The dry samples were cut into pieces weighing around 50 mg and immersed in a vial with phosphate buffered saline, PBS (pH 7.4). The samples were placed in a shaking incubator at 37 °C. Buffer was changed weekly. All tests were performed in triplicate. After the periods of evaluation, the samples were removed from the buffer, gently dried with a filter paper and

153 washed three times with deionized (DI) water and then dried in an oven for 24 hours at 60 °C

154 and immediately weighed.

155 The degradation ratio was determined according to the following equation:

156

157 $W_l = [(W_f - W_i/)W_i]*100$; were:

158

159 W_l= weigth loss, W_i= Initial dry weight, W_f= dry weight after time degradation,

160

161 The results shown are the average of 3 replica.

162

163 **Table 1**

164 Amounts of reactants for the synthesis of HPUC series.

Sample	PLGAdiol (g)	NCO/OH 2:1:1 DMPA/PLGAdiol (wt%)	CS (g)	Swelling Capacity (wt%)
HPUC1	0.25		0.75	1200
HPUC2	0.5	7	0.5	1800
HPUC3	0.75		0.25	2000

165

166 **2.6. In vitro and in vivo assay**

167 This study was published and approved by the Health Sciences Commission of the School

168 of Medicine of the Pontifícia Universidade Católica do Rio Grande do Sul - PUCRS under

169 number 6236, and also by the Committee on Ethics in the Use of Animals - PUCRS,

170 registered under number 15/00469. All procedures and techniques used during the animal

171 experimentation are in accordance with the regulation proposed in the Brazilian Guidelines

172 for the Care and Use of Animals for Scientific Purposes, by the National Council for the

Control of Animal Experimentation (CONCEA, 2013) and the Ethics Committee for Use of Animals of PUCRS (CEUA).

2.6.1. Mononuclear Bone Marrow Fraction isolation (BMMNCs)

After euthanasia the donor animals were dissected and bone marrow was aspirated from the long bones. The aspirated material was placed in falcon tubes containing RPMI medium and was transferred to another tube containing FicollPaque (Histopaque 1119, Sigma Aldrich) and centrifuged at 400 g. The mononuclear cell layer was collected and washed with Dulbecco's Phosphate Buffered Saline (DPBS). Viability was then determined using the trypan blue exclusion method. The same procedure was used for the tissue regeneration assay.

2.6.2. Cell culture

The cytotoxicity assay was performed with the adherent cell fraction. The cells were cultivated until confluence in a 75 cm^2 cell culture flask containing Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% of Fetal Bovine Serum (FBS), gentamicin (0.025 g/L and streptomycin/penicillin (0, 1 g/L) in humid atmosphere at 37 °C with 5% of CO_2.

2.6.3. MTT assay

Cytotoxicity was evaluated according to ISO 10993-5, evaluating the mitochondrial function by tetrazolium salt reduction (MTT). The sample extracts were prepared in DMEM. After sterilization with ethanol, the HPUC membranes were washed by immersion in sterile Milli Q water three times and then once in DPBS and DMEM without FBS, immersed in DMEM medium for 24, 48 or 72 hours and incubated with stirring in a humid atmosphere at 37°C with 5% of CO_2. DMEM medium was used as negative control and copper sulfate (0.1 g.mL^{-1}) in DMEM medium as positive control. All membranes were cut to a size of 3 cm^2 and were incubated in 5 ml DMEM to allow the extraction. The BMMNCs suspension was adjusted and seeded at a density of 0.5 x10^4 cell per well onto 96-well culture plates and incubated for 24 hours to allow cellular adhesion. The cells were then incubated for 24 h with DMEM and after that, the reading was performed. The absorbance values obtained were normalized by negative control with 100% viable cells. All the tests were performed in triplicate.

2.6.4. Direct contact

The cells were seeded at a density of 5 x 10^4 cells per well onto a 24-well culture plate. The membrane was cut into a small circle (11-mm diameter) and placed in the middle of a well and incubated for 24 hours, 48 hours and 72 hours in humid atmosphere at 37°C with 5% of

CO_2. Cell viability was evaluated by the Live/Dead Cell Viability Assay on an Inverted Phase Contrast Fluorescence Microscope (Thermo Fischer Scientific) according to the manufacturer's instructions.

2.6.5. Animal model of diabetic ulcers

60 day-old adult female Wistar rats weighing between 200 and 300g were given water and food ad libitum. Tail blood was collected at time 0 to evaluate the glycemic levels. All animals were fasted for 8 hours prior to diabetes induction, which was by an intraperitoneal injection of Streptozocin (70 mg/kg). Seven days after the streptozotocin injection the blood glucose levels of the animals were measured by the Accu-Check Active kit (Roche Diagnostics GmbH). The animals with blood glucose levels above 250mg/dL were considered diabetic. After glucose measurement, the animals were divided into two groups as described below. The animals submitted to the simulated ulcer procedure were given anesthesia of 80% Ketamine and 20% Chlorpromazine, followed by a dorsal incision of about 6mm diameter with a scalpel to simulate an ulcer wound.

2.6.6. Experimental Design and Treatment of Groups

In the *in vivo* evaluation for minimizing the use of animals, only HPUC1 membrane was selected because it is less fragile than HPUC2 and HPUC3 because its water sorption capacity is smaller while cytotoxicity results were similar in the three samples (see below) . The 16 rats were divided into 2 groups: Group 1 (control) (n=8): diabetic animals, with a wound, without treatment; Group 2 (n=8): diabetic animals with HPUC and BMMNCs transplanted onto the wound site. After the formation of the wound, 1×10^6 cells of the mononuclear bone marrow fraction were transplanted into each animal in Group 2 by injection into the edge of the wound. After this procedure, the HPUC was positioned and sutured onto the wound. The animals were evaluated histologically in two different periods of time: 7 and 14 days after membrane implantation.

2.7 Histopathology of diabetic mice wounded skin tissues

Samples obtained from the histological analyses of the wound were removed 7 and 14 days after wound formation. To obtain the sample, the wounded area was removed from the animals backs after euthanasia in the CO_2 chamber. All the samples were fixed in neutral buffered formalin and embedded in paraffin wax. Each section was 4µm thick and stained with hematoxylin-eosin (H&E). The analysis of the epithelial tissue and the depth of the epithelial cell-covered wound were considered. The analysis of connective tissue was

considered for the study of the inflammatory process, measured by the type and quantity of inflammatory cells and presence / absence of fibrosis, using H&E staining. The analyses were performed by two blinded examiners, previously instructed by a pathologist and calibrated by the kappa coefficient test.

2.8. Macroscopic wound closure analyses

The closure of the wounds was measured by comparing with ImageJ software the images recorded during the experiments. The formula used to measure the wound regeneration rate was as follows:

RWA = (DA)/(OWA)*100; were:

RWA: Residual wound área (%), DA: Detected Area, OWA: Original Wound Area.

2.9. Statistical Analysis

Quantitative data was expressed as mean ± standard deviation. To compare the regeneration of the wound between the groups, an unpaired t test was perfomed at each time to compare the difference in wound closures. Values with $p < 0.05$ were considered statistically significant. All the statistical analyses were performed with the statistical GraphPad Prism.

3. Results and discussion

3.1 Synthesis of hydrogel membranes

The chitosan waterborne polyurethane was obtained in two stages as shown in the reaction scheme in Figure 1. The first (i) step consisted of the synthesis of the polyurethane prepolymer (PU prepolymer) with different weights of the soft segment poly (lactide-co-glicolide) diol, with HDI and DMPA as a chain extensor. In the second (ii) step, the HPUC was obtained by crosslinking reaction of the PU prepolymer with chitosan during membrane formation.

The synthesized membranes to be used as wound dressings were successfully obtained as shown in Figure 1. However, the membrane (HPUC3) with the highest amount of prepolymer (75%) became more brittle than the others (Figure S1 in Supplementary Material). This could have been due to the membrane presenting a phase separation, which may have contributed

266   to the immiscibility of the components and making it brittle (Tg 15 °C, data not shown). This
267   was not observed in the other two membranes.

268
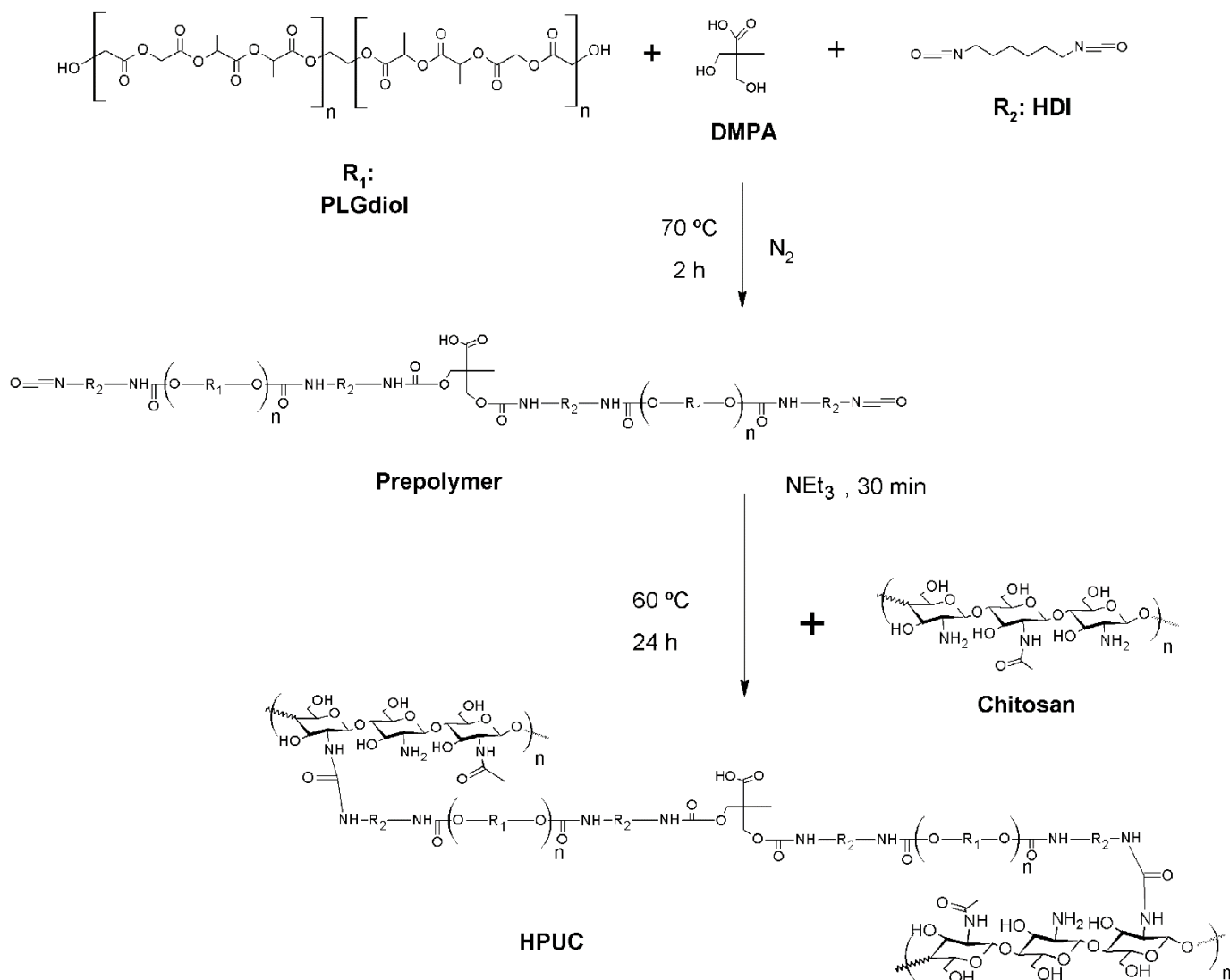
Fig. 1. Synthesis route of the HPUC membranes.

271

3.2. Swelling

273   Chitosan was soluble in acetic acid 1% while the prepolymers were soluble in DMSO, acetone
274   and CHCl3. The membranes were not soluble in the solvents used to solubilize the
275   prepolymer or in acetic acid 1%, proving the formation of crosslinked networks. Sample
276   weight in immersion in liquid water stabilizes after 48 hours (See Figure S2 in Supplementary
277   Material). The water sorption capacity at 48 hours for the different samples is larger than
278   1000% measured on dry basis (Table 1). The network swelling capacity confirms that,

contrary to what could be expected, the membrane with the highest hydrophobous component, HPUC3, had the highest water uptake. The water content measured at 48 hours of immersion time thus fell from about 2000 to 1200 when the PLGA content was reduced from 75% to 25% (samples HPUC3 and HPUC1).The figure does not show a curve for pristine chitosan because it is water soluble, since the amine groups remain protonated after dissolution in the acidic medium. Hydrogel equilibrium water content is described by the Flory Rhener Equation (Flory & Rehner, 1943), which shows that swelling increases with a favorable water-polymer segment interaction parameter and with the higher average molecular weight between crosslinks in the network, i.e. with a reduced crosslinking density. The higher PLGA/CHS ratio should yield a less favorable average interaction parameter between PU soft segments and water. The fact that swelling capacity nevertheless increases could be due to less efficient crosslinking with the presence of a significant number of unreacted carboxyl end groups in PU prepolymer.

### 3.3. FT-IR of chitosan polyurethane.

The spectra of original chitosan and polyurethane based on chitosan with different soft segment contents are shown in Figure 2A.

The chitosan spectrum shows the typical large peaks in the 3340 cm-1 region relative to the stretching of the OH and NH2 groups, 2872-2920 cm-1 to CH2 stretch, 1151 cm-1 for C –O –C of the chitosan ring. The appearance of the peaks relative to urea and urethane bonds can be observed in the case of polyurethane prepolymer and the networks. The membrane formed has two hard segments due to the urea and urethane bonds formed between the diisocyanate groups and NH_2 and OH chitosan groups and PLGA/DMPA, respectively (Subramani, Park, Lee, & Kim, 2003). These urethane bond peaks appear in the 1730-1746 cm-1 region (C=O stretch), 1027-1074 cm-1 (C-O stretch) and urea bonds at 1616 -1625 cm-1 (C=O stretch), 1589 cm-1 (NH bending) and 1254 – 1260 cm-1 (C-N stretch). As found by other authors, the urea bond formation (-NHCONH-) occurs because the preferential reactivity of NCO groups with NH_2 groups is much more reactive than the OH groups (S. H. Chen et al., 2012; Pérez-Limiñana, Arán-Aís, Torró-Palau, Orgilés-Barceló, & Martín-Martínez, 2005; Velazquez-Morales, Le Nest, & Gandini, 1998).

In the supplementary data, Figure S3, the FTIR spectra of the synthesized PLGAdiol and the PU prepolymer are shown. Although is observed an overlapping in the urethane and urea absorption region the peaks around 1600-1750 cm-1 are distinguishable. The presence of

the absorption band at 1625 cm-1, which is ascribed to hydrogen-bonded urea groups (Marchant, Zhao, Anderson, & Hiltner, 1987; Zhang, Ren, He, Zhu, & Zhu, 2007) and is absent in the prepolymer is slightly different in the networks with varying composition, showing that sample composition affects the reaction mechanism.
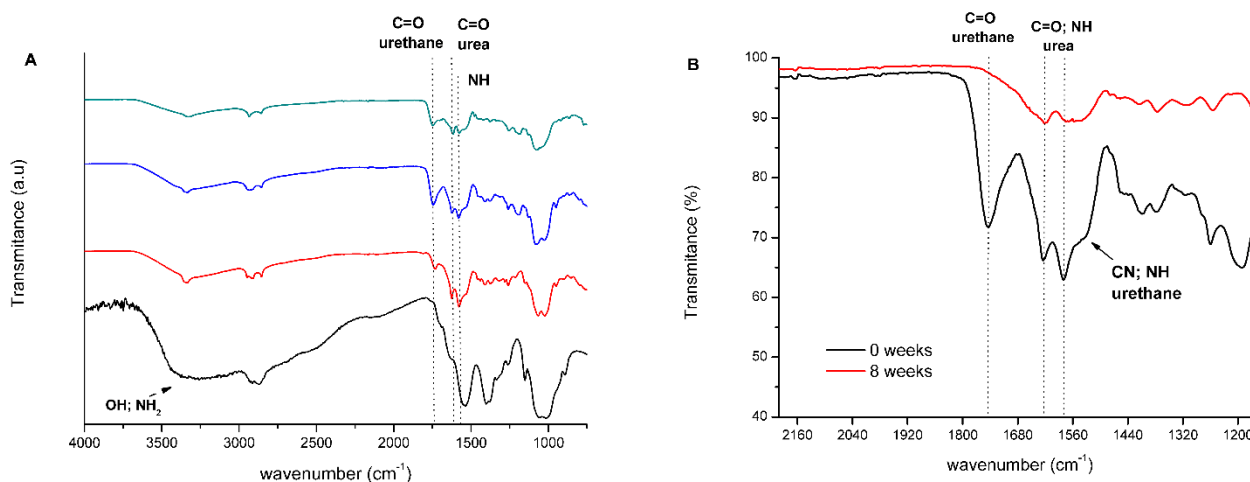
Fig. 2. FTIR spectrum of membranes. (A) Membranes before the degradation test. Arrows and lines indicate the main groups related to crosslinking between the prepolymer PU and CHS (in black: CHS, red: HPUC1, blue: HPUC2 and green: HPUC3). (B) Membrane HPUC2 after 8 weeks immersion in PBS 1x buffer.

3.4. Solid state ^{13}C-NRM

Figure 3 shows the solid state NRM spectra of chitosan and HPUC membranes with 25% (HPUC1), 50% (HPUC2) and 75% (HPUC3) soft segment. In chitosan the peaks in Figure 3 are those of C1 (δ104.8), C4 (δ81.5), C5 and C3 (δ75.1), C6 and C2 (δ57.2), and CH3 (δ22.6), in agreement with other studies (de Moura, Aouada, & Mattoso, 2008; Heux, Brugnerotto, Desbrières, Versali, & Rinaudo, 2000). For HPUC membranes we observed novel peaks, which have been reported as the chitosan-PU crosslinking for HPUC1 peaks: δ174.82, δ159.27, δ99.49, δ73.24, δ60.60, δ41.65, δ29.50, δ17.34; for HPUC2: δ176.77, δ159.76, δ101.92, δ73.24, δ61.09, δ41.65, δ29.98, δ16.86 and HPUC3: δ174.34, δ159.27, δ102.40, δ74.40, δ60.60, δ42.13, δ30.64, δ23.13. The carbonyl (CO) shifts are far from the field (155-210 ppm) and generally weak due to slow relaxation. For urea and urethanes the shifts are 170-180 and 150-160, respectively. The peak of ^{13}C spectra in the urea group occurs at a

higher magnetic field than those in the urethane group (Zhang, Cheng, & Hu, 2003)□. The displacement, found in HPUCs around $\delta174$, is related to urea bounds between $-NH_2$ groups of chitosan and -NCO groups of PU prepolymer, while $\delta159$ is related to both urethane bonds -NHCOO- of polyester and DMPA in the soft and hard segments, respectively (Barikani et al., 2010; Leventis et al., 2010)□.
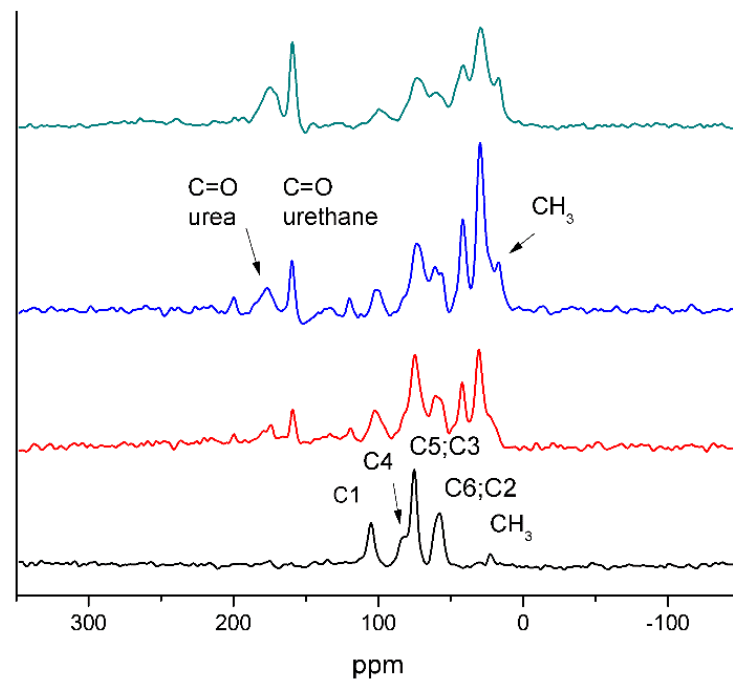

Fig. 3. Solid state ^{13}C-NRM spectrum of membranes. Arrows indicate the main shifts related to crosslinking between the prepolymer PU and CHS (in black: CHS, red: HPUC1, blue: HPUC2 and green: HPUC3).

3.5 Thermogramymetric analysis

Figure 4B show the derivative weight loss (DTG) curves of chitosan and HPUC membranes. In the chitosan and HPUC TG curve we observed the first thermal event in the 40-140°C range, which indicates a weight loss of less than 6% attributed to the evaporation of residual water from the polymer (de Britto & Campana-Filho, 2007)□. The second thermal event, after 140 °C, is due to the overlapping of the degradation of chitosan and PLGA chains. Thermal degradation of the chitosan used in this study takes place in the 187-372 °C range (with a maximum in the DTG plot at 268 °C) and is attributed to the thermal decomposition of chitosan chains (Abused et al., 2014; Neto et al., 2005; Zawadzki & Kaczmarek, 2010)□. For HPUCs, thermal decomposition in the TG curve occurs in a broader temperature range

between 142 and 502°C. The overlap of the different phenomena can best be seen in the DTG curve (Figure 4B).

The peak appearing at the lower temperature is attributed to the degradation and weight loss of the hard and soft segments of the urethane groups (the intensity of this peak increases with increasing PLGA content, as expected). This peak appears at temperatures lower than that of pure chitosan degradation (the maximum in DTG curve appears at ~235ºC). On the high temperature side of this peak, in the chitosan degradation temperature range a shoulder can be seen indicating the decomposition of chitosan chains in the network. The highest temperature peak in the HPUCs is relative to the weight loss of the hard segment of the urea bond, since the energy of the urea bond is larger than that of the urethane bond, the degradation peak in urea appears at higher temperatures (García-Pacios, Costa, Colera, & Martín-Martínez, 2011). Table 2 gives the chitosan and HPUC thermal data. HPUC membranes had a lower onset temperature, HPUC1> HPUC2> HPUC3, with PLGA incorporated in the chitosan structure, which could be associated with changes in the intermolecular interactions between polysaccharide chains resulting in reduced polymer thermal stability.
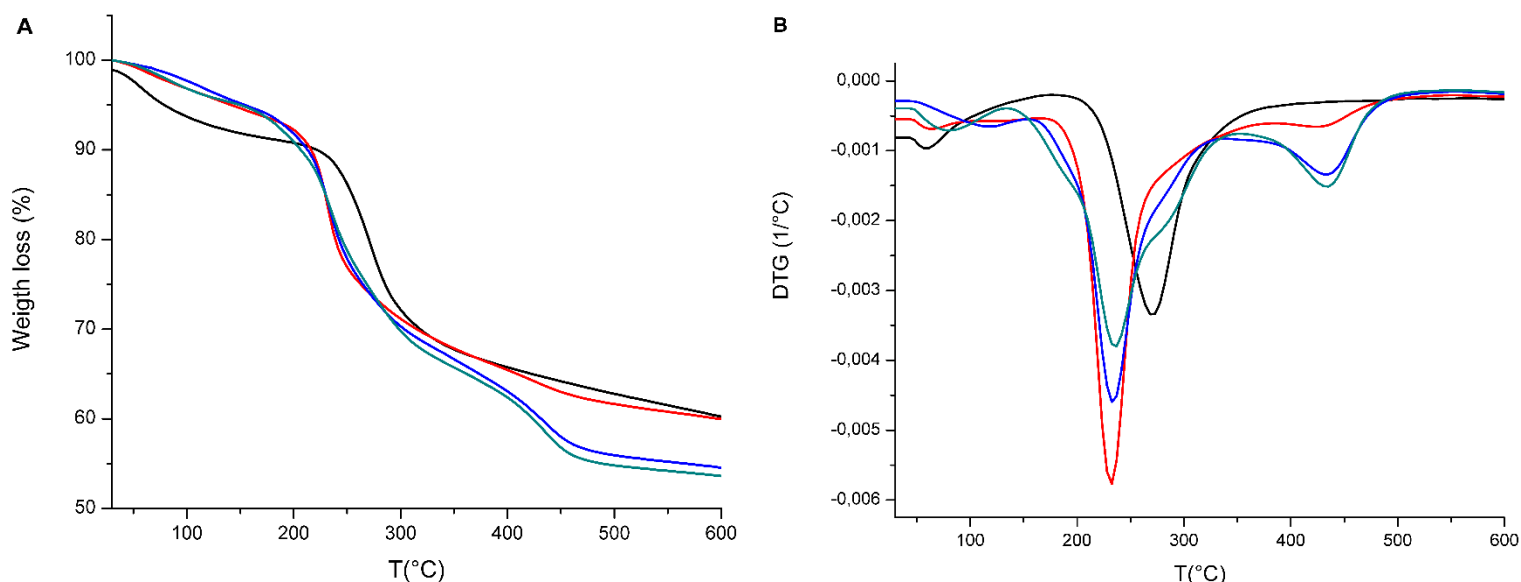
Fig. 4. Graphs of the relative weight loss curves (A) and derived weight loss (B) of chitosan membranes and HPUCs (in black: CHS, red: HPUC1, blue: HPUC2 and green: HPUC3).

Table 2

Maximum degradation temperature of CHS and HPUCs.

Sample	Temperature range ºC	Second Stage T$_{max}$ (ºC)	Third Stage T$_{max}$ (ºC)	Weight loss %
CHS	187-372	268		30
HPUC1	171-476	232.5	426	36
HPUC2	151-507	232.5	435	40
HPUC3	142-507	237	435	41

3.6. Degradation in PBS medium

Figure 5 shows the degradation curves in an aqueous solution (PBS 1x pH 7.4) for up to eight weeks. There is a significant mass loss at the end of the eight weeks in the HPUC2 and HPUC3 samples, 87.0% and 90.5% respectively, compared to 56.4% for HPUC1 and 53.2% for CHS. The findings show that membrane weight loss has two different mechanisms. Since the membranes were not neutralized at the end of the synthesis, one could expect that the chitosan chain amine groups would remain protonated and that the chitosan would be slowly dissolved in the aqueous solution. Thus, the pure chitosan film presents a continuous weight loss of up to 53.2%, which cannot be explained by hydrolysis. This delivery of chitosan chains by dissolution in the aqueous medium is prevented in the HPUC hydrogels by the polymer network structure. Nevertheless, HPUC2 and HPUC3 membranes can degrade by nearly 100% in eight weeks. Since cleavage of chitosan chains by hydrolysis does not take place in the degradation times of our experiments, we hypothesize that the cleavage of the PLGA chains and urea linkages liberates chitosan chains that are susceptible to being dissolved in the medium, since they are protonated, contributing to the weight loss (See the schema in Figure S4 of supplementary material).

FTIR analysis support this assumption. After eight weeks of degradation of the HPUC membranes (Figure 2B) it is clear the disappearance of the peaks characteristic of the PU

regions between 1500-1150 cm-1, and the strong band at approximately 1745 cm-1 corresponding to a C = O bond of ester groups showing the degradation of the PLGA segment. In addition, the original position of the amide peak at 1579 cm-1 (C-N and N-H) moves to 1575 cm-1 (Figure 2B). It is also possible to visualize the displacement of the 1625 cm-1 peak for 1619 cm-1 corresponding to the carbonyl bond in the urea groups (C = O amide I) indicating degradation of the rigid segment ((Marcos-Fernández, Abraham, Valentín, & Román, 2006; Pretsch, Jakob, & Müller, 2009), the same can be seen in the 1530 cm-1 region referring to PU urethane group bound to the CHS chains.

The high swelling capacity of the HPUC network, even for the largest PLGA content, contributes to the hydrolysis process. PLGA chains are exposed to water molecules in the swollen sample and thus the hydrolysis of the ester groups is faster than in bulk PLGA which is highly hydrophobous.

Thus, we propose that the fast, and nearly complete degradation of the HPUC hydrogels is due to the combination of the loose of the degradation fragments of PLGA chains together with the delivery of CHS chains detached from the network structure.
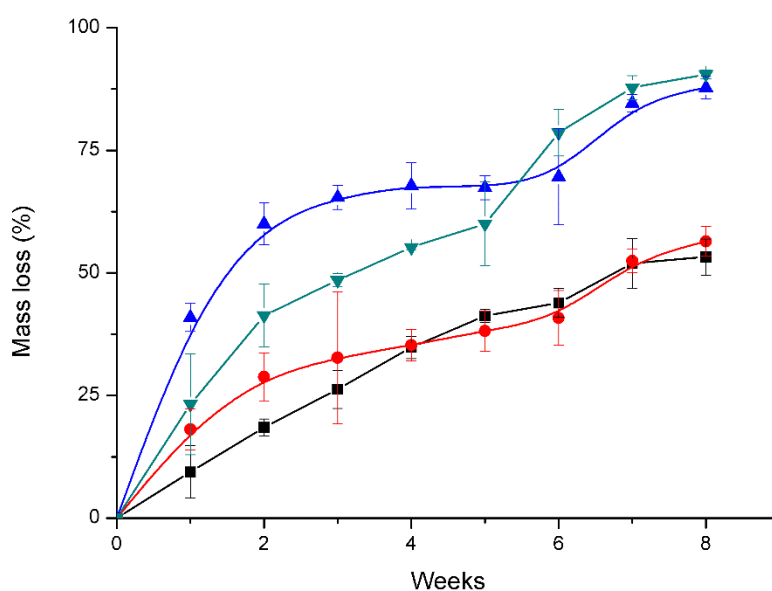
Fig. 5. CHS and HPUC mass loss curves after up to eight weeks: ■ CHS, ● HPUC1, ▼HPUC2 and ▲HPUC3. All values are expressed as mean ± standard deviation (n=3).

3.7. In vitro cytotoxicity analysis of HPUC membranes

To select the best membrane to continue the *in vivo* assay we evaluated cellular viability by mitochondrial metabolic activity. For the indirect cytotoxicity analysis, the adherent BMMNCs were cultured with membrane extracts obtained after immersion of the membranes for periods of 24, 48 and 72 hours. The viability of the cells cultured in direct contact with HPUC membranes in the same time periods was assessed with a live/dead test with fluorescence microscopic evaluation. In all the exposure periods in the indirect contact experiment the viability was of all the membranes above 80% of the negative control (Figure S5 in Supplementary Material)

Considering the results obtained for HPUC3 degradation time and brittleness in the swelling experiments, we opted for HPUC1 and HPUC2 membranes to continue the evaluation. The HPUC1 and HPUC2 networks were analyzed in direct contact using the live/dead fluorescence assay, which provides information on physical cell properties (cellular membrane integrity) and biochemical intracellular esterase activity (Masson-Meyers, Bumah, & Enwemeka, 2016). No cytotoxicity effect was detected (see images in Figure 6), indicating that the cells produced esterase (green fluorescence), which is an indicator of viable cells, while the red fluorescence that indicates cell death is rarely observed. The images show the cells well spread out on the culture plate, while the morphology and cell numbers are quite similar to those of the controls.

3.8. HPUC plus BMMNCs accelerated wound repair in diabetic rats

Considering the high costs of treating patients with ulcers in the lower limbs, and the high morbidity and mortality rate caused by this type of wound, the research for new treatments is necessary. Diabetic patients affected by ulcers in the lower limbs go through a prolonged inflammatory phase during tissue regeneration, with persistence of a pro-inflammatory profile, moving from an acute to a chronic state. This prolonged inflammation is directly related to the precarious healing of these wounds, leading these individuals to recurrent infections, and when not properly treated at an early stage, can lead to amputation. Our study aimed to identify whether the use of a biological dressing associated with mononuclear bone marrow fraction cells from rats would be able to improve the regenerative response in ulcerous wounds in diabetic rats.

From an intraperitoneal use of streptozotocin, it was possible to establish an animal model of diabetes. After the model was established, we were able to create a wound on the animals'

backs to simulate an ulcer wound, simulating the ulcer shown in lower limbs from diabetic individuals. From the results, it was observed that the group of animals treated with the HPUC combined with the injection of BMMNCs in the interphase between the tissue and the HPUC membrane (Group 2) showed significantly better results in terms of healing in the first 7 days after the formation of the wound compared to the control group (Figure 7). During the first few days, we observed that the animals that had the HPUC plus BMMNCs on the wound had a cleaner and drier wound, when compared macroscopically with the animals that did not receive the treatment. This suggests that the membrane achieved hemostasis of the wound, supporting the formation of the fibrin matrix and cell infiltration (Eming, Martin, & Tomic-canic, 2014; Oberyszyn, 2008).

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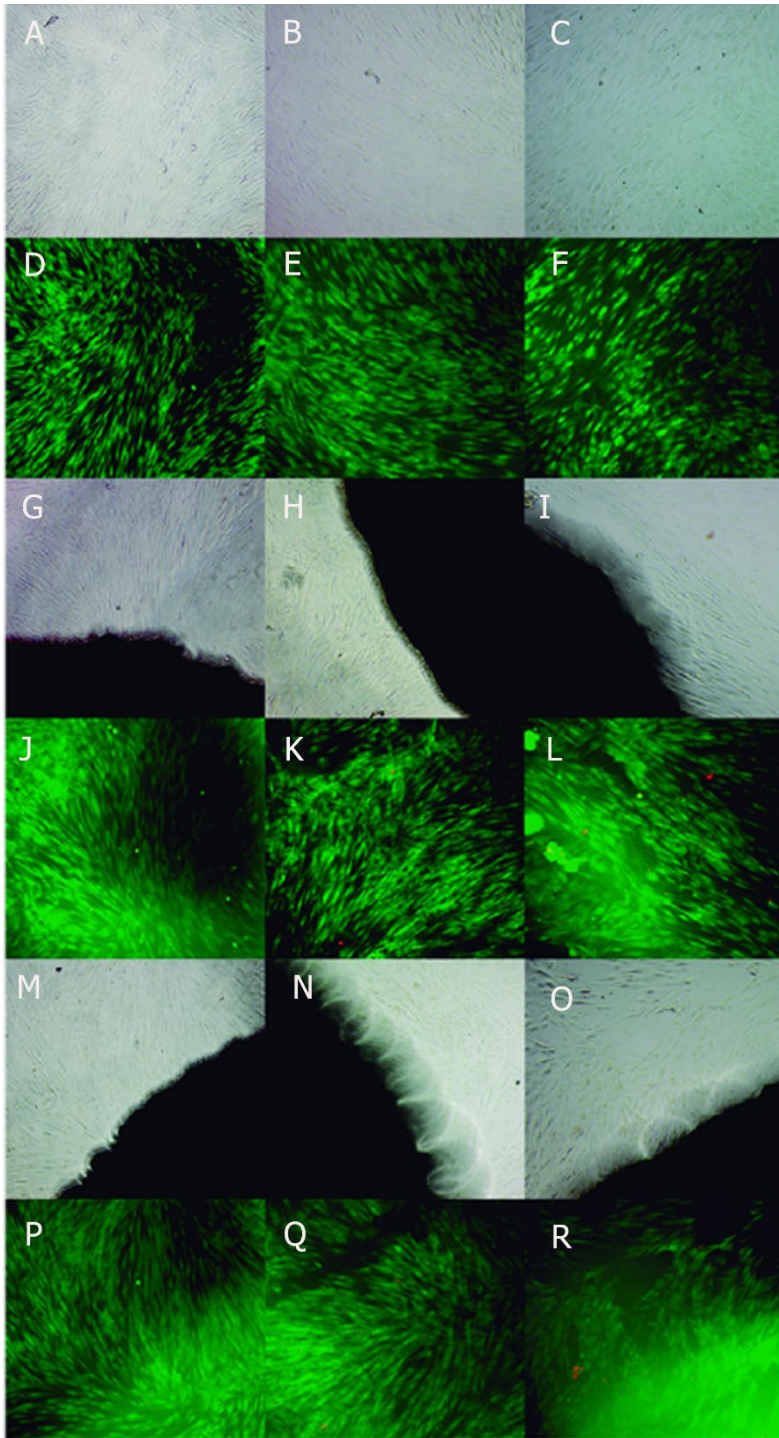
Fig. 6. Representative fluorescent image of MSCs: (**A – F**) Control, (**G– M**), HPUC2, (**N – S**) HPUC1. Live cells were stained with calcein AM (green) using a FITC filter and dead cells were stained with Ethidium homodimer (red) and captured by an PI filter. All images were acquired after 24, 48 and 72 hours. Magnification 100x.

In addition, the animals treated with the BMMNCs plus the biomaterial showed a significantly better regeneration rate than those from the control group after 14 days, with a significant reduction in wound size.

The use of bone marrow-derived mesenchymal cells enhances tissue regeneration, significantly reducing the wound size in rat diabetes ulcer models using streptozotocin. In addition, complete wound healing time was also significantly lower in the mesenchymal cell treated group (Kuo et al., 2011). The combination BMMNCs and HPUC provided wound healing in just 14 days (wound closure >90%). Although previous studies in the literature with different biomaterials showed a significant improvement of wound healing, they required longer times and a more intense inflammatory process (Ahmed et al., 2018; Sayed et al., 2016). The macroscopic analysis of wounds treated with these membranes revealed a considerably smaller wound bed than those in the control group.
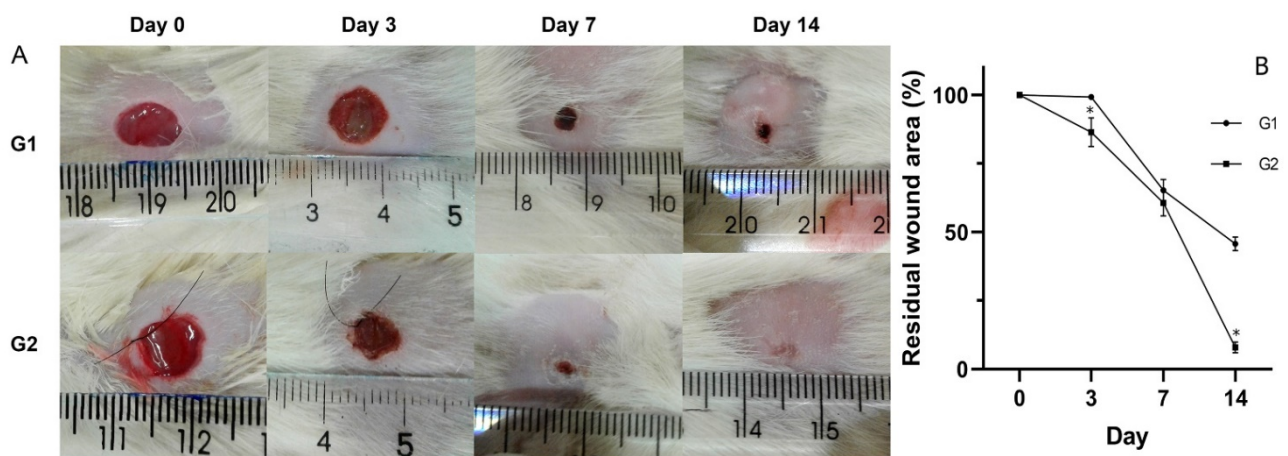
Fig. 7. Representative image in the effects of HPUC1 and BMMNCs on wound healing in diabetic ulcer model. (A) Macroscopic observations in wound areas in the control (G1) and wound healing contraction induced by HPUC1 plus BMMNCs (G2). (B) Residual wound area. The results are expressed as mean ± standard deviation (n= 6). (*) indicates significant difference, $p < 0.05$.
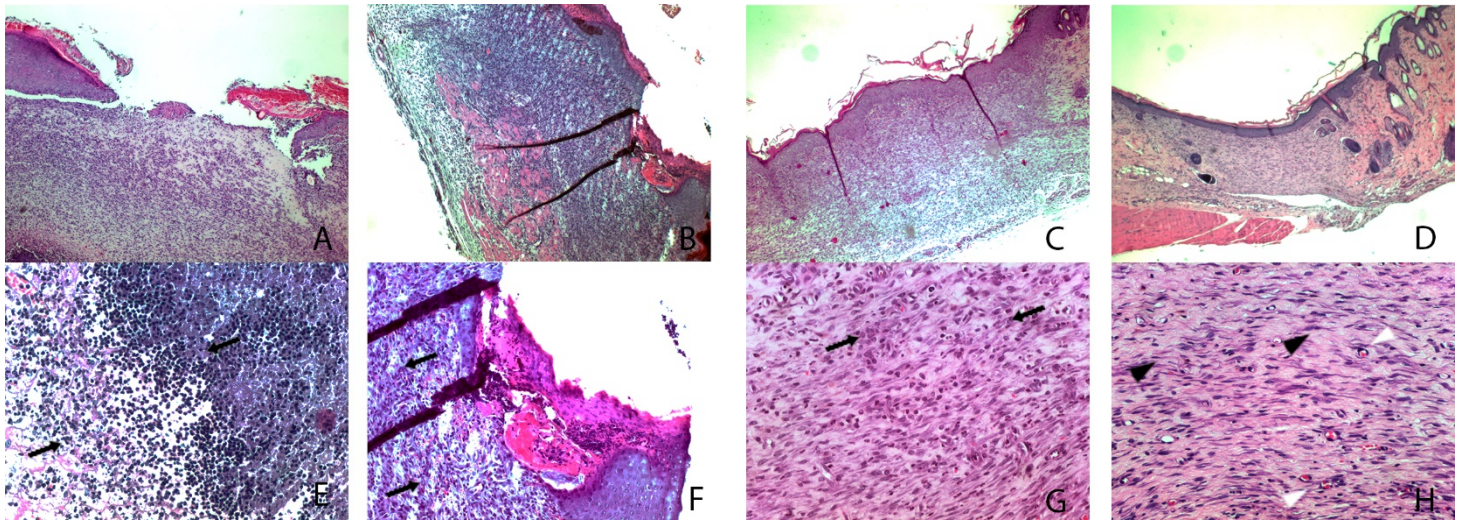
3.9. Histological wound healing by H&E staining

To evaluate the role of BMMNCs in the remodeling and speed of tissue regeneration when transplanted with a HPUC membrane, we collected the samples from the wound region in the diabetic rats for histological analysis on days 7 and 14. On day 7 it was observed a

505 noticeable difference on wound closure from the treated group (Figure 8) compared to the
506 non-treated group.

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Fig. 8. Representative histologic analysis of the wound region. Tissue samples were
collected on day 7 (**A, E, C, G**) and 14 (**B, F, D, H**). H&E staining showed severe
nflammation in the control group (**A, B, E, F**) and wound healing and re-epithelialization (**C,
D, G, H**) in the HPUC1 plus BMMNC group. Black arrows indicate inflammatory cell
infiltrate, black arrowhead fibroblasts and white arrowhead indicate blood vessels.
Magnification 4x in A, E, C, D, and 20x in B, F, G, H.

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Unlike Group 2 (HPUC + BMMNCs), Inflammatory cell infiltrate was observed in the control
group and there were no signs of epithelial regeneration. In Group 2, moderate inflammatory
infiltrate was visible, together with wound closure with epithelization. On day 14 remodeling
of the wound was clearly seen in Group 2 with organized connective tissue and
neovascularization, indicating successful healing. This was not found in the control group.

Initial inflammation at the beginning of healing is critical for the regeneration period, which
dictates the second phase of wound regeneration, with reduced inflammation remodeling and
culminating in wound closure (Koehler, Brandl, & Goepferich, 2018)□. All these processes
involve several cell types. The mesenchymal cells in the BMMNC fraction of bone marrow
aspirates may have a positive effect on the anti-inflammatory process for their differentiation

and paracrine effects (L. Chen, Tredget, Wu, Wu, & Wu, 2008; Park, Ahn, Sung, Ahn, & Chang, 2018). Another important point was the large HPUC membrane water absorption capacity (shown in swelling tests). Better dressing water uptake could lead to different forms of exudate retention, which may cause changes in cytokine levels and growth factors on the wound surface and faster cicatrization (Yamane et al., 2015).

4. Conclusions

This paper describes a study of synthesized chitosan/PLGA networks, which were found to present continuous degradation in aqueous solution. The hydrolytic degradation of polyurethane blocks liberated chitosan chains that dissolved in the aqueous solution. We confirmed that this biomaterial has low cytotoxicity and supports cell proliferation. The synthesis was based on unmodified chitosan and new crosslinkers consisting of a low molecular weight polyester polyurethane with NCO terminations, enabling crosslinking with free amine of chitosan. The use of BMMNCs helped to close the wounds, reduce inflammation and improve neovascularization around the wound. The results show that the synthesized hydrogel plus BMMNCs is suitable for use as a dressing for diabetic wounds and has a potential use in tissue engineering.

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