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TISSUE ENGINEERING CONSTRUCTS AND CELL SUBSTRATES

Original Research

Injectable porcine bone demineralized and digested extracellular matrix—PEGDA hydrogel blend for bone regeneration

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Abstract

Extracellular matrix (ECM) has a major role in the structural support and cellular processes of organs and tissues. Proteins extracted from the ECM have been used to fabricate different scaffolds for tissue engineering applications. The aims of the present study were to extract, characterize and fabricate a new class of hydrogel with proteins isolated from pig bone ECM and combine them with a synthetic polymer so it could be used to promote bone regeneration. Porcine bone demineralized and digested extracellular matrix (pddECM) containing collagen type I was produced, optimized and sterilized with high pressurized CO_2 method. The pddECM was further blended with 20% w/v polyethylene glycol diacrylate (PEGDA) to create an injectable semi interpenetrating polymer network (SIPN) scaffold with enhanced physicochemical properties. The blend tackled the shortfall of natural polymers, such as lack of structural stability and fast degradation, preserving its structure in more than 90% after 30 days of incubation; thus, increasing the material endurance in a simulated physiological environment. The manufactured injectable hydrogel showed high cytocompatibility with hOb and SaOs-2 cells, promoting osteogenic proliferation within 21 days of culture. The hydrogel had a high compression modulus of 520 kPa, low swelling (5.3 mg/mg) and millimetric volume expansion (19.5%), all of which are favorable characteristics for bone regeneration applications.

1 Introduction

Bone is one of the most important structural entities in the body. Bone defects due to either degenerative diseases or traumatic injuries impose a great cost on public health in both developed and developing countries and are expected to increase with an aging population [1–3]. To overcome bone loss and to promote bone repair, different materials in combination with surgical interventions are available to aid the regeneration process including the use of autologous bone grafts, allografts, xenografts, alloplasts or synthetic products [4–8].

However, current materials used for bone regeneration have limitations such as the availability of donor site, morbidity of the surgical procedure, risk of immune rejection, and associated high costs [9]. Thus, artificial materials offer an alternative source for the growing needs of bone regeneration therapies [7, 10]. In this context, polymers are interesting substitutes that have been actively investigated in the field of regenerative medicine. Polymeric hydrogels hold great potential as a regenerative biomaterial for a number of biological applications, such as the possibility to control the cellular response, acting as a carrier for drugs/active molecules, and versatility to be combined with other materials [11, 12]. Additionally, hydrogels usually have high water content, which resembles to some extent the tissues extracellular matrix (ECM). Nonetheless, a common shortfall of hydrogels is their lack of inherent biomechanical stability, which affects their clinical use particularly in larger and less contained bony defects [13, 14].

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The ECM is a complex, highly organized assembly of macromolecules and signaling factors produced by the local cells of each tissue or organs [15]. It exists in an active balance with the microenvironment [16], influencing the cellular activity and responses [17]. Biological scaffolds derived from ECM from urinary bladder [18], dermis [19] and bone itself [20] have shown to promote the regeneration of different tissues, including heart [21], cartilage [22] and bone [20]. These findings suggest that proteins extracted from the ECM could be a good alternative to fabricate matrix derived scaffolds to regenerate structurally similar tissues [12].

In bone regeneration, has been reported that type I collagen can stimulate bone host cell differentiation, adhesion, mineral deposition [23, 24], it can control the turnover of fibril formation and cell-matrix interaction [25]. Nevertheless, low biomechanical strength of natural polymers such as collagen and their limited retention in physiological conditions are obstacles for their application in hard tissue regeneration [26].

Two main approaches have been used to address the low biomechanical properties of natural hydrogels. The first one is the physical addition of micro and nano-fillers and the second is the formation of an interpenetrating polymeric network (IPN). The heterogeneous nature of physical mixtures limits the application of micro and nano-fillers in tissue engineering. Therefore, the formation of an IPN comprised of bone ECM proteins with a synthetic polymer is hypothesized as an effective approach to address the shortcomings of collagen based scaffolds used in bone regeneration. One common strategy for blending two independent polymers with different chemical compositions is the semi-IPN approach. A semi-IPN is produced when a linear polymer penetrates a second polymer hence, generating a network that is partially interlocked and does not have chemical bonds between them [27, 28].

Hydrogel blends constructed with natural and synthetic polymers have been previously fabricated to strengthen the mechanical properties and the stability in physiological conditions. For example, hydrogels made of methacrylated gelatin (GelMa) and poly lactic-ethylene oxide fumarate (PLEOF) demonstrated favorable physicochemical and biological properties [29]. Furthermore, adding PEGDA to PLEOF has shown an increase in the mechanical strength of the hydrogel blend from 125 to 570 kPa [29, 30]. However, synthetic materials or their derivatives lack of intrinsic biological activity to promote tissue regeneration.

The aims of this study were to develop a biological approach to extract and preserve the intrinsic bone-specific biological activity of pig bone ECM proteins and combine them with PEGDA in order to fabricate an osteoconductive injectable SIPN hydrogel with favorable physicochemical properties for bone regeneration. To this end, we hypothesized that an optimized multi-step method including demineralization, defatting, digestion, and dialysis can be used to extract porcine bone ECM proteins, while a synthetic polymeric blending will assure the physicochemical stability and micro-architecture.

2 Materials and methods

2.1 Bone fragments preparation

To prepare bone fragments, fresh pig heads were harvested from adult porcines. The mandibles were removed and the gonial angle was separated to obtain bone segments. The segments were further washed with a solution containing phosphate-buffered saline (PBS) with 0.1% (w/v) Penicillin/Streptomycin (pH = 7.4). Under refrigerated conditions, the bone segments were cut with a circular saw into fragments smaller than $4 \times 4 \times 4\ mm^3$ and washed with PBS two times for 4 h in an orbital stirrer to remove the excess of blood. The clean fragments were first freeze dried for 24 h and then immersed in liquid nitrogen for a few minutes to embrittle the bones. A commercial grinder was used to produce pulverized bone powder.

2.2 Demineralization and lipids removal

Bone powder granules were demineralized by modifying previously developed methodologies [20]. Briefly, the bone powder granules were demineralized in a washing solution of 0.5 N HCL ($25\ ml\ g^{-1}$) at 37 °C under constant agitation (250 rpm) in an orbital stirrer for 8 h changing the washing media every hour. The particles were always suspended in motion in the acid solution to prevent settling during the process. The degree of demineralization of the bone granules was investigated by standard radiography at different time points (2–24 h). The remaining HCl solution was removed by centrifugation (4000 rpm) for 15 min and the resultant material was carefully washed with sterile Milli Q water 4 times to remove the remaining acid.

To remove lipids and fat contents and aid with the decellularization, the demineralized bone powder was further washed with a solution of chloroform and methanol (1:1 volume ratio) for 4 h at 4 °C. Subsequently, the defatted material was washed several times with sterile Milli Q water until no traces of the chloroform/methanol solution could be detected. The material was then freeze dried overnight and later weighted for further processing.

2.3 Digestion and sterilization

Digestion of the demineralized and defatted bone powder granules was performed by modifying previously developed

methods [20, 31]. Briefly, a 1 mg ml^{-1} Pepsin (Sigma-Aldrich, pepsin from porcine gastric mucosa lyophilized powder, ≥2.500 U/mg protein) solution in 0.01 N HCl was added to 10 mg of material (i.e., 10 mg of material in 1 ml of 0.01 N HCl with 1 mg of pepsin). The resulting suspension was stirred in glass beakers at RT for 24, 48 and 72 h to compare different set times of digestion. The suspension was left on the stirrer until it formed a viscous solution with no visible fragments of remaining granular material. This was subsequently referred as porcine bone demineralized and digested extracellular matrix (pddECM).

To eliminate the HCl used in the digestion phase and neutralize the pH, the pddECM was dialyzed against 5 L sterile PBS pH 7.4 in 3.5 KDa MW cut-off SnakeSkin™ dialysis tubing (Thermofisher scientific) for 48 h, changing the washing media three times every 12 h. Next, to confirm the decellularization of the material, representative samples were freeze dried, fixed in 4% paraformaldehyde (PFA) neutral buffered for 2 h and next exposed to a gradual series of ethanol dehydration (30%, 50%, 70%, 90%, 100%) for 1 h each to then infiltrate the samples with (Technovit 8100®) overnight. Finally, infiltrated samples were embedded in resin (Technovit 8100) and sections of 3 µm were stained with Toluidine Blue to identify the presence of visible cell nuclei under optical microscope (Olympus BX60). Quantification of DNA content was conducted by an adaptation of previously reported methods [20, 32]. Briefly, nuclear and mitochondrial DNA were isolated from pig bone powder and pddECM, using the DNeasy Blood & Tissue Kit (QIAGEN GmbH, Germany) following the manufacturer's instructions. DNA concentration was assessed using a NanoPhotometer® (Implen) by UV absorption spectrophotometry at 260 nm. DNA detection and quantification were performed by using mitochondrial [32] and genomic primers (designed Ensembl for β-Actin; ACTB-201; ENSSSCT00000048365.1).

Prior to the analysis a standard curve was prepared ($R^2 = 0.999$) with pig bone DNA to acquire an accurate reading. The standard curve was established with at least 6 known DNA concentration points. DNA amplification and quantification were performed using Stratagene Mx3000P and the enzymes SensiFAST™ SYBR® Lo-ROX Kit (Bioline) accordingly to the manufacturer's instructions.

To investigate the sterility of the material, microbial loaded pddECM samples treated with high pressurized CO_2 at 38 °C and 190 bar were tested for contamination [33]. Mesophilic bacteria and yeasts and molds presences were analyzed before and after the treatment using a standard plate count technique [31]. Before each experiment, a high concentrated poly-microbial extract was obtained. Briefly, poly-microbial juice was incubated at 25 °C for 18 h to increase the initial microbial load in a range of 10^4–10^5 colonies forming units per ml (CFU/ml) following the methodology of a previous report [34]. For each analysis, 100 µL of the solution containing pddECM loaded with the poly-microbial juice were spread on plate count agar for mesophilic bacteria and yeast and mold agar for yeasts/molds, using negative controls of sterile MilliQ Water (MQW) and positive controls of poly-microbial juice alone. The plates were incubated at 35 °C for 72 h in triplicates. Microbial-free and equilibrated pddECM with a final pH of 7.4 was then stored at −20 °C until required.

2.4 SDS-PAGE characterization of the pddECM

Previous to the electrophoresis analysis, pepsin digested samples of pddECM were quantitated by the Bradford protein assay. For each analysis 15 µg of protein sample was loaded onto 12.5% SDS-PAGE gels according to the Laemmli method [35]. Gels included 15 µg molecular weight standards (MW) (BioRad) and 15 µg Collagen Type I (Sigma-Aldrich) as a positive control. Samples were first suspended in 20% sample buffer (150 mM Tris-HCl, 4% (w/v) Sodium Dodecyl Sulfate (SDS), 40% (v/v) glycerol, 0.5% (w/v) Bromo Phenol Blue (BPB) pH = 7 and 6% (v/v) 2-mercaptoethanol) then denatured for 3 min at 100 °C. Gels were run at 120 V and fixed twice in solution containing 50% (v/v) methanol, 10% (v/v) acetic acid and 40% (v/v) Milli Q water then stained with Coomassie brilliant blue G-250 for 3–4 h. Destaining took place using fixing solution diluted 8 times until the bands were clearly detectable.

2.5 2D electrophoresis characterization of the pddECM

The pddECM sample subject to isoelectric focusing (IEF) was solubilised in modified surfactant solution (MSS) buffer containing 7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 30 mM Tris, 2% (w/v) sulfobetaine, 1% (v/v) Triton X-100, 5 mM TBP and 0.1% (w/v) Bromophenol Blue and incubated at RT for 30 min to allow full protein reduction. Samples were alkylated with the addition of 15 mM iodoacetamide (IAA) and then incubated at RT for 20 min. Prior to focusing, 2% (v/v) IPG buffer (pH 3–10 or pH 4–7 depending on the strip), 2% (v/v) DeStreak reagent (GE Healthcare Life Sciences) were added. A protein mixture of 200 µg/180 µl was used to rehydrate 7 cm pH 4–7 Immobiline DryStrip Gels (IPG strips) (GE Healthcare Life Sciences) and 600 µg/380 µl MSS for 18 cm linear pH 3–10 IPG strips overnight at RT. IPG strips were focused at 20 °C on the Multiphor II Electrophoresis System (GE Healthcare Life Sciences) for a total of 12 kV h for 7 cm IPG strips and 60 kV h for 18 cm IPG strips. After the first dimensional focusing, IPG strips were equilibrated in equilibration buffer containing 6 M urea, 2% (w/v) SDS, 50% (v/v) glycerol,

0.375 M Tris and 5 mM TBP for 15 min at RT. This was followed with a second equilibration in equilibration buffer containing 2.5% Iodoacetamide for another 15 min [36]. Strips were placed onto 8–15% gradient polyacrylamide gels for separation of proteins in the second dimension on the mini-PROTEAN Tetra-Cell (Bio-Rad) (7 cm IPG strips) and Ettan DALT*six* Electrophoresis System (GE healthcare life sciences) (18 cm IPG strips). Small format gels containing 7 cm strips were run at 100 V while large gels containing 18 cm strips were run overnight at 30 mA/gel. After electrophoresis, gels were fixed in fixing solution, stained with Coomassie Brilliant Blue G-250 and destained in MQ diluted fixing solution (8:1). Protein spots chosen to be excised for identification by mass spectrometry had to be consistently expressed in all gels for further processing.

2.6 Liquid chromatography mass spectrometry characterization of the pddECM

Liquid chromatography mass spectrometry (LCMS) analysis was conducted using the LC coupled to a QExactive Plus Orbitrap mass spectrometer (Thermo Fisher Scientific, Scoresby). Approximately 100 protein spots per sample were chosen. The selected spots or bands were excised from the gel and distained in a 60:40 solution of 40 mM NH_4HCO_3 (pH 7.8)/100% acetonitrile (MeCN) for 1 h. Gel pieces were vacuum-dried and the rehydrated with a 12 ng/µl trypsin (Promega) solution at 4 °C for 1 h. Excess trypsin was removed and gel pieces were covered with 40 mM NH_4HCO_3 and incubated overnight at 37 °C. Peptides were concentrated and desalted using C_{18} Zip-Tips (Millipore, Bedford MA) as per the manufactures instructions. Peptides were resuspended in 50 µl 3% (v/v) acetonitrile/0.1% (v/v) formic acid, and briefly sonicated. Samples were then separated by nano-LC using an Ultimate 3000 HPLC and auto-sampler system (Thermo Fisher Scientific, Scoresby) coupled to an in-house fritless nano 75 µm × 40 cm column packed with ReproSil Pur 120 C_{18} stationary phase (1.9 µm, Dr, Maisch GmbH, Germany). LC mobile phase buffers were comprised of A: 0.1% (v/v) formic acid and B: 80% (v/v) acetonitrile/0.1% (v/v) formic acid. Peptides were eluted using a linear gradient of 5% B to 40% B over 30 mins and then 95% B wash over 1 min at a flow rate of 250 nL/min. Column voltage was set at 2300 V and the heated capillary at 275 °C. Positive ions were generated by electrospray and the Orbi-trap operated in data-dependent acquisition mode. A survey scan of 350–1550 m/z was acquired (resolution = 70,000, with an accumulation target value of 1,000,000 ions) with lock-mass enabled. Up to 10 of the most abundant ions (>1.7e5 ions), with charge states ≥ +2 were sequentially isolated and fragmented and target value of 100,000 ions collected. Ions selected for MS/MS were dynamically excluded for 20 s. The data were analyzed using Proteome Discoverer vr 2.0 (Thermo) and Mascot vr 2.4 (Matrix Science, London). The search parameters included the following variable modifications: oxidized methionine and carbamidomethyl cysteine. The enzyme was set to trypsin precursor with a mass tolerance of 10 ppm while the fragment tolerance was 0.1 Da. The database was the NCBnr (September 2015) restricted to *Sus Scrofus Domestica* taxonomy.

2.7 Hybrid pddECM/PEGDA hydrogel blend fabrication

The pddECM/PEGDA hydrogel blend was fabricated using SIPN method. In this method, a known mass ratio of pddECM and low molecular weight PEGDA (Sigma Aldrich, Mn 700) was used to cross-link the blend by the addition of a crosslinking agents ammonium persulfate (APS) and ascorbic acid (vitamin C). The mixtures A. 0.1% (w/v) pddECM (pH 7.4) containing 5% APS and B. 20% w/v PEGDA containing 3% ascorbic acid were loaded into a double chamber syringe with a spiral mixer tip (Intra-oral syringe, 3M ESPE).

2.8 Crosslinking time

The vial tilting method was used to measure the gelation time of the pddECM/PEGDA hydrogel blends fabricated with different concentrations of pddECM and PEGDA [31]. Hydrogels containing a mix of pddECM (0.2%, 0.1% and 0.05% w/v) and PEGDA (40%, 20 and 10% w/v) were assessed. The concentrations of 3% ascorbic acid and 5% APS were kept constant as it was optimized in previous studies [30]. In this analysis, the polymeric solutions with different formulations were dispensed via injection into 2 ml vials and left at RT. The gelation point was recorded at the time when there was no flow upon inverting/tilting the vial.

2.9 Millimetric volume expansion

Dimensional volume expansion of pddECM/PEGDA hydrogel solution (~1000 mm^3) with different concentrations of pddECM and PEGDA was measured inside standardized 2 ml vial tubes when subjected to PBS soaking. The volume variations were recorded in cubic millimeters (mm^3) at 1, 2, 3 and 7 days post-incubation. Additionally, free discs of at least 7 mm in height and 12 mm in diameter (~1000 mm^3), were fabricated and placed inside 25 ml tubes containing PBS. Similar to the contained experiment, the dimensional volume expansions were measured at 1, 2, 3 and 7 days post-incubation to determine the behavior of volume variation in a free space. The measurements were performed with an electronic caliper and the volume was calculated by the following equation: Hydrogel volume =

$\pi \times r^2 \times h$ where r is the radius of the hydrogel and π is "pi" and h is height.

2.10 Mass swelling ratio

The swelling ratio of fabricated pddECM/PEGDA and PEGDA control hydrogels was measured gravimetrically. To this end, samples were weighed before and after their incubation in PBS for 1, 2, 3 and 7 days. Mass swelling is reported as the wet weight to the dry mass ratio determined by the following equation: Swelling ratio $= (W_s - W_d)/W_d$, where W_s is the swollen weight and W_d is the dry weight of the hydrogel after freeze drying the initial specimen.

2.11 Release of pddECM fraction from the hydrogel blend

The efficiency of pddECM/PEGDA hybrid hydrogels (~1000 mm^3) to retain pddECM within their structures was assessed based on the degree of pddECM release after 1, 4, 7 and 10 days of soaking in 10 ml of PBS in simulated physiological conditions. At different time intervals, up to 100 µl of solution were transferred into Eppendorf tubes and the protein concentration of each sample was measured using Bradford´s standard protein-assay method. To measure the remaining pddECM in the hydrogel structure, the samples were liquefied by increasing the temperature and quantified with Bradford's assay at 10, 20 and 30 days of incubation.

The degree of pddECM release was calculated based on the concentration of released protein with respect to dried weight of samples. Prior to this analysis a calibration curve was prepared ($R^2 = 0.999$) by analyzing standard solutions of pddECM to acquire an accurate reading. A standard curve was established with 6 known protein concentration solutions.

2.12 In vitro degradation assay

The pddECM/PEGDA hydrogel (~1000 mm^3) degradation rate was determined in the presence of 400 units/ml of collagenase A (Sigma) mixed with Hank's balanced salt solution and in PBS. The samples were incubated at 37 °C in orbital shaking for 30 days changing the respective solutions every 3 days.

The pddECM/PEGDA hydrogels were freeze dried and weighed after 5, 10, 15, 20, 25 and 30 days post-incubation under simulated physiological conditions. The degradation was calculated using the following equation: Degradation $\% = W_i - W_n/W_i \times 100$, where W_i and W_n are the dry weight of the sample before and after n days of incubation [29] in collagenase A and PBS, respectively.

2.13 Mechanical testing

Uniaxial compression tests in an unconfined state were conducted to assess the mechanical strength of hybrid hydrogels. Instron® (Model 5543, Illinois Tool Works Inc) with a 1 kN load cell and a loading rate of 0.5 mm/min was used [29]. The compression (mm) and load (N) were collected using Bluehill® 3 software. The compressive modulus was obtained as the tangent slope of stress–strain curve in the linear region, between 10 and 20% strain level.

2.14 Cell activity

Human osteoblasts (hOb) and human osteosarcoma cells line SaOs-2 were used to assess the biocompatibility/cytocompatibility and osteoconductivity of the hydrogels. The hObs were cultured in hOb basal medium supplemented with 1% hOb growth supplement, 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin. SaOs-2 were cultured with DMEM culture medium, supplemented 10% bovine calf serum (BCS) with 1% penicillin/streptomycin and L-glutamine at 37 °C in a humidified atmosphere with 5% of CO_2. The pddECM/PEGDA hydrogels (~1000 mm^3) were injected with a syringe (3 M ESPE) into 24-well tissue culturing polystyrene plates forming cylindrical structures. Subsequently, hObs and SaOs-2 were seeded onto the crosslinked hydrogels at a density of 2×10^5 cells/well. Hydrogels containing 20% w/v PEGDA without pddECM were injected and seeded at the same conditions as described above to serve as controls. All conditions were assessed in triplicates.

After 7, 14 and 21 days cell viability was determined by using a live/dead assay kit (Invitrogen) according to the manufacturer's instructions. Briefly, live cells were stained green color whereas dead cells were red. Live and dead cells were observed using an inverted fluorescence microscope (Leica DMI 6000B- U). Cell proliferation was determined using MTS (Promega) [3-(4,5-dimethylthiazol-2-yl)-5-(3-crboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay. Briefly, the cells seeded on pddECM/PEGDA were incubated in a mixture of fresh media (250 ml) and MTS solution (50 ml). The samples were kept in a CO_2 incubator at 37 °C for 1 h allowing MTS to react with metabolically active cells and produce water-soluble formazan product, which can be measured quantitatively using a microplate reader (Bio Rad 680) at 490 nm [29].

Alkaline phosphatase (ALP) (Abnova) was used to detect the production of phosphate using P-nitrophenyl phosphate (pNPP) as a colorimetric substrate, which can be measured quantitatively at 415 nm wavenumber. This test was used to investigate the osteoconductivity of the fabricated hydrogels. Migration of hOb and SaOs-2 cells was measured on fixed slices of seeded hydrogels. Briefly, the hydrogels were fixed with 4% PFA for 2 h and next exposed to a gradual

series of ethanol dehydration (30%, 50%, 70%, 90%, 100%) for 1 h each before incubating in absolute ethanol overnight. The hydrogels were measured with a caliper to record the dimensions and later embedded in resin polymer (Technovit®). Resin cuts of 3 µm were stained with Toluidine Blue and the presence of nuclei was identified under optical microscope (Olympus BX60).

Cell morphology was examined by scanning electron microscopy (SEM). Hydrogels cultured with cells for 21 days were fixed in 2.5% glutaraldehyde and 4% PFA in 0.1 M phosphate buffer for 8 h to then be triple washed with PBS to clean the samples from the remaining fixative. The hydrogel structures were then dehydrated by critical point drying by washing them with ethanol sequence (30%, 50%, 70%, 90%, 95%, 100%) to remove water. Then 1% Osmium tetradoxide in 0.1 M PBS was added 1 h at RT as a secondary fixative to then be dried by CO_2 in a critical point dryer (Leica EM, CPD 300). These samples were platinum coated using (Emitech K550X) machine prior to SEM analysis (Zeiss ULTRA Plus).

2.15 Statistical analysis

The results were compared by applying the statistical 1-way analysis of variance (ANOVA) followed by *post hoc* Bonferroni test with the aid of STATA software V 13.0. $p < 0.05$ was considered statistically significant and is marked in figures with $*p < 0.05$. The number of samples for each experiment was $n = 5$ unless otherwise described.

3 Results

3.1 Preparation of pddECM

To prevent the denaturation of the protein extract, the mineral phase of the bone powder was removed using an optimized time exposure in 0.5 N HCl. Radiographic imaging demonstrated that full demineralization of the bone powder granules can be achieved within 8 h (Supplementary Fig. 1).

After demineralization, lipids and fats were removed with chloroform/methanol solution. Decellularization was also achieved with this procedure, and it was confirmed with Toluidine blue staining demonstrating the absence of cell nuclei. Additionally, DNA quantification showed a genomic DNA of 0.38 ng dsDNA per mg initial dry weight and 0.08 ng of mitochondrial dsDNA per mg initial dry weight, resulting in a content compatible with implantable biomaterials (Supplementary Fig. 2 and Table 1) [37].

Investigation of the enzymatic digestion time in buffered pepsin showed complete digestion within 24 h of pepsin treatment, demonstrating no significant difference among samples after this time point.

Sterilization of the pddECM was achieved by using high pressurized CO_2 at 38 °C and 190 bar for 3.5 h. Samples of pddECM co-cultured with poly-microbial juice and sterilized with this method demonstrated the aseptic condition of pddECM (0 CFU/ml) when tested for mesophilic bacteria and yeasts and molds after 72 h of incubation (Supplementary Fig. 3).

3.2 Biological characterization of the retrieved pddECM

The characterization of the pddECM by SDS-PAGE revealed high MW proteins compatible with β, α_1 and α_2 collagen type I fragments, visible at a range of 125–210 kDa and α_1 dimer fragments at 101 kDa. The protein profile of pddECM at 24, 48 and 72 h did not show any change, with collagen type I fragments similarly distributed at each time point as shown in Fig. 1a. Comparison of the pddECM sample with the collagen type I control showed no major visible differences between the protein bands although the control showed more bands under 38.5 kDa. To identify the proteins found in the SDS-PAGE, a 2D electrophoretic profile of pddECM was performed. IPG strips of 18 cm pH 3–10 revealed around 40 spots with the majority of proteins located within the pH 4–7 and 29–150 kDa range as depicted in Fig. 1b. Narrow range pH 4–7 IPG strips were then used to improve, and narrow down the separation of the desired proteins resulting in 60 pddECM spots detectable between 21 and 150 kDa, where the majority of proteins were focused at the acidic end of the gel as shown in Fig. 1c.

The spots from the pH 3–10 and pH 4–7 focused gels were cut and analyzed by LCMS. From a total of 93 proteins analyzed, the largest group was classified as collagen type I α_1 chain and collagen type I α_2 precursor, thus classifying the overall protein extract as a collagen type I.

3.3 Fabrication of pddECM/PEGDA hydrogel blend

Hydrogels of pddECM/PEGDA blend were fabricated by loading each solution in a double chamber syringe with a spiral mixer tip; thus, facilitating the blending and crosslinking of the polymers. Concentrations of 0.05 and 0.1% w/v of pddECM and 10, 20 and 40% w/v of PEGDA were utilized. The constructed pddECM/PEGDA hydrogel blends showed stability to increased temperatures elevated to 37 °C. The effects of the concentration of pddECM and PEGDA on the crosslinking time, physicochemical properties and retention of pddECM in the structure of hydrogels were further investigated.

3.4 Crosslinking time

The gelation time of different concentrations of pddECM and PEGDA was investigated by the vial tilting method.

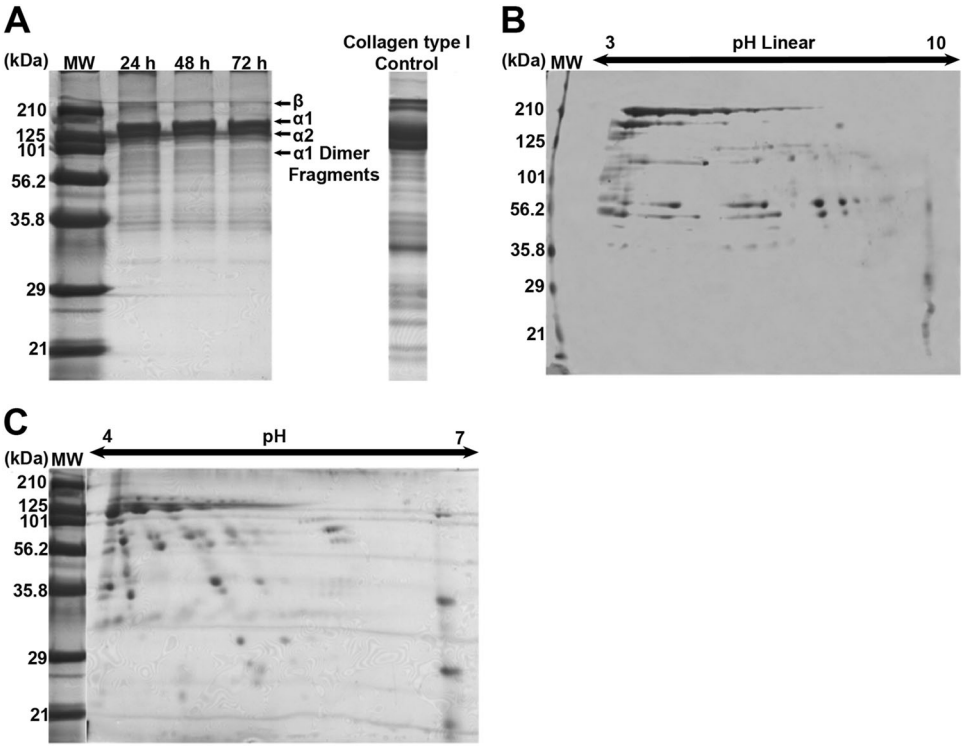

Fig. 1 1D and 2D electrophoresis of pddECM sample. **a** SDS-PAGE 12.5% resolving gel, 15 µg pddECM sample digested with pepsin at 24, 48 and 72 h, Collagen Type 1 (Sigma-Aldrich) control (15 µg). **b** 2D electrophoresis of pddECM (600 µg) focused on an 18 cm linear pH 3–10 IPG strip. **c** 2D electrophoresis of pddECM (200 µg) focused on a 7 cm pH 4–7 IPG strip (kDa kilo-daltons; MW molecular weight)

Crosslinking of 0.1% w/v pddECM/20% w/v blend took place after 90 s post mixing, a time similar to the 20% w/v PEGDA control hydrogels. Reduction of pddECM concentration to 0.05% w/v produced gelation at 170 s, while reduction of PEGDA to 10% w/v showed an increase in the crosslinking time to 230 s. Reducing the concentration of both polymers resulted in an increase gelation time of 220 s. On the other hand, doubling the concentration of both polymers also increased the gelation time to 290 s (Fig. 2).

3.5 Impact of pddECM and PEGDA concentrations on the volume expansion of pddECM/PEGDA hydrogel blend

Two different methods were used to assess the volume expansion of the pddECM/PEGDA hydrogel blends. The first test involved different concentrations of pddECM/PEGDA hydrogels injected in 2 ml vials and soaked in PBS. The results of this test (Fig. 3a) showed a dimensional expansion of 109.08 mm^3 (10.9%) for 0.1% w/v pddECM/20% w/v PEGDA blends after 7 days of incubation. An expansion of 540 mm^3 (54%) for 0.1% w/v pddECM/10% PEGDA blends was demonstrated, while a 1000 mm^3 (100%) was experienced in the 0.05% w/v pddECM/20% w/v PEGDA hydrogels at day 7. Reducing both polymer concentrations to 0.05% w/v pddECM/ 10% w/v PEGDA produced an increase in 446 mm^3 (41.3%) at day 7. Controls of 20% w/v PEGDA experienced small volumetric changes

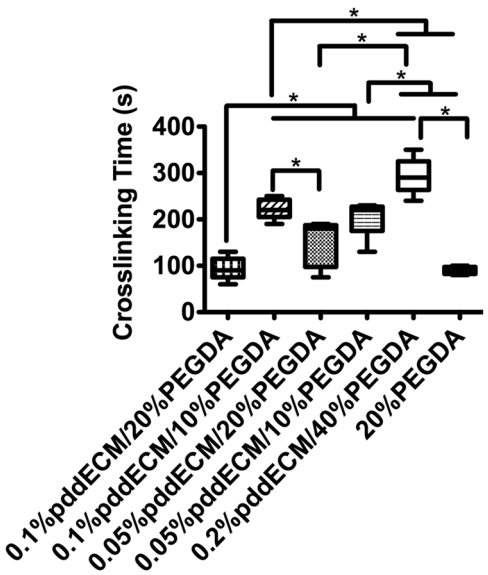

Fig. 2 Crosslinking time of pddECM/PEGDA blends. Gelation time assessed by vial tilting technique. *$p < 0.05$; $n = 5$

of 170 mm^3 expansion (16.7%) during the entire period of observation.

The second test evaluated discs of different pddECM and PEGDA concentrations soaked in PBS (Fig. 3b–f). The results demonstrated an expansion of 195 mm^3 (19.5%) for 0.1% w/v pddECM/20% w/v PEGDA hydrogels by day 7. Hydrogels with 0.05% w/v pddECM/20% w/v PEGDA increased 257 mm^3 (26.2%) at day 7, while 0.1% w/v

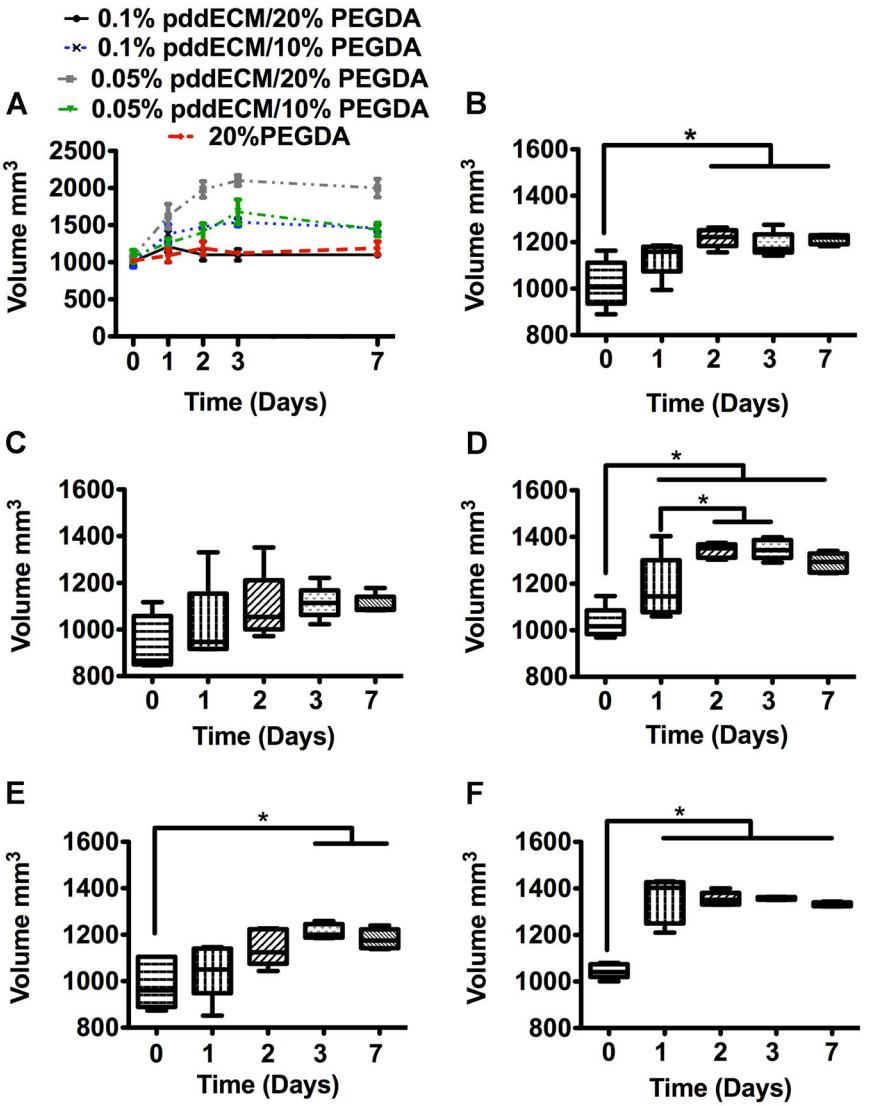

Fig. 3 Volume expansion of pddECM/PEGDA hydrogel blends **a** volume change of 0.1% w/v pddECM/20% w/v PEGDA blend fabricated inside standardized 2 ml vial tubes and soaked in PBS. Free hydrogel discs of **b** 0.1% w/v pddECM/20% w/v PEGDA **c** 0.1% w/v pddECM/10% w/v PEGDA **d** 0.05% w/v pddECM/20% w/v PEGDA **e** 0.05% w/v pddECM/10% w/v PEGDA **f** 20% w/v PEGDA. (*$p < 0.05$; $n = 5$; *Time 0 as base line*)

pddECM/10% w/v PEGDA experienced a slight decrease of 177 mm^3 (17.7%). Finally, reducing both concentrations to 0.05% pddECM w/v and 10% PEGDA w/v resulted in a volume change of 192 mm^3 (20.1%) at day 7. All the experiments were carried out for a period of 30 days; however, no recordable changes were measured beyond day 7.

The analysis confirmed that at least 0.1% w/v pddECM and 20% w/v PEGDA were required to provide adequate crosslinking, uniform structure and stability. Therefore, only optimized 0.1% w/v pddECM and 20% w/v PEGDA pddECM/PEGDA hydrogel blends were generated and used for all further studies.

3.6 Mass expansion of pddECM/PEGDA hydrogels

To determine the gravimetric mass swelling ratio of 0.1% w/v pddECM and 20% w/v PEGDA hydrogels, samples were weighed before and after their incubation in PBS at 37 °C at different time points The results indicated swelling from 4.7 mg/mg right after the 1st day of incubation, followed by a further increase to 5.5 mg/mg on day 2. By day 3, the mass ratio decreased again to 5.3 mg/mg to then remain stable to day 7 (Fig. 4a).

3.7 Release of pddECM fraction from the optimized pddECM/PEGDA hydrogel blend

The leaching of pddECM fraction from the optimized 0.1% w/v pddECM/20% w/v PEGDA hydrogels was measured to examine the final retention of pddECM within the hydrogel blend. As shown in Fig. 4b, approximately 206 ± 5.33 µg (20.6%) of pddECM were leached out from the hydrogel after day 1. At day 8, an accumulated release of 811.2 µg (81.1%) was detected. However, after the day 9, no pddECM was detectable in

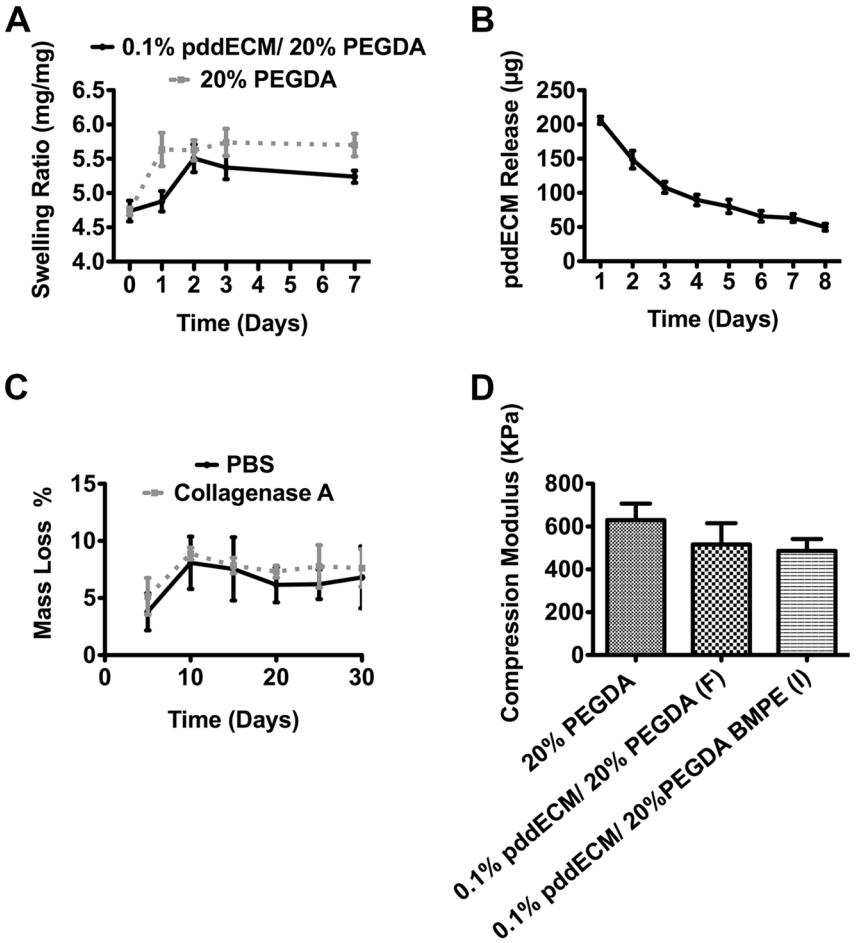

Fig. 4 Physicochemical properties of optimized 0.1% w/v pddECM/20% PEGDA hydrogel blends. **a** Swelling ratio of hydrogel discs expressed as the swollen weight over the dry weight. **b** Release of pddECM from the 0.1% w/v pddECM/20% w/v PEGDA hydrogels blends incubated in PBS. **c** Cumulative mass loss of 0.1% w/v pddECM/20% w/v PEGDA blends over 30 days of incubation in PBS and Collagenase A solution. **d** Compressive modulus of 20% w/v PEGDA control, freshly prepared 0.1% w/v pddECM/ 20% w/v PEGDA (F) and 0.1% w/v pddECM/20% w/v PEGDA after 9 days of PBS incubation (I) (F freshly prepared hydrogel; I incubated hydrogel)

the incubation media anymore. Residuary pddECM in the hydrogel was determined by liquefying the remnant structure. Bradford's assay revealed 188.8 ± 37.8 µg (18.88%) of remnant pddECM thus, demonstrating retention of pddECM in the hydrogel.

3.8 Degradation rate of the optimized pddECM/ PEGDA hydrogel blend

The degradation rate of the optimized pddECM/PEGDA hydrogels within 30 days of incubation was assessed in PBS, used as a neutral media, and in collagenase A to investigate the enzymatic effect on the pddECM fraction. The hydrogels showed an initial degradation of 3.7 ± 1.6% after 5 days of incubation in PBS. In contrast, the pddECM/ PEGDA blend in collagenase A solution experienced a degradation of 5.1 ± 1.6%. At day 10 after incubation, the original mass of the hydrogels was reduced by 8.1 ± 2.3% in PBS and 8.8 ± 0.5% in collagenase A solution respectively, representing the highest percentage of mass loss during the observation period. At the day 30, a degradation of 6.8 ± 2.7% in PBS and 7.6 ± 1.6% in collagenase A solution was observed (Fig. 4c).

3.9 Mechanical properties of the optimized pddECM/ PEGDA hydrogel blend

The mechanical testing revealed the highest compression modulus in 20% w/v PEGDA controls with a mean of 630 kPa, followed by freshly prepared 0.1% w/v pddECM/ 20% w/v PEGDA hydrogels with a mean of 520 kPa. After 9 days of incubation in PBS the 0.1% w/v pddECM/20% w/v PEGDA hydrogels still showed a mean compression modulus of 490 kPa. Nonetheless no statistical difference was seen between these results (Fig. 4d).

3.10 Cytocompatibility of optimized pddECM/PEGDA hydrogels

Cytocompatibility of the optimized 0.1% w/v pddECM/ 20% w/v PEGDA hydrogel blend was compared with 20% w/v PEGDA controls. The present findings confirmed the adhesiveness and cytocompatibility of pddECM/PEGDA blend providing a favorable scaffold for hOb and SaOs-2 cells to proliferate and grow for more than 21 days. Live and dead assay showed that after 21 days of culturing the number of living cells seeded onto the pddECM/PEGDA

hydrogels was significantly higher compared to PEGDA controls (Fig. 5). Colonization patterns of hOb and SaOs-2 cells seeded in 20% PEGDA were random, while the cells appeared preferentially located around pores in the pddECM/PEGDA hydrogel blends.

The MTS assays showed an increase in viable cells for both groups of hOb and SaOs-2 in the pddECM/PEGDA hydrogels group at 21 days of culturing compared to PEGDA controls (Fig. 6a, b). The ALP test showed an increase cell enzymatic activity at day 21 for pddECM/PEGDA hydrogels, while the cell enzymatic activity in PEGDA hydrogels remained constant (Fig. 6c, d). The difference may be explained by the presence of porosity and less density in the pddECM/PEGDA hydrogels, which facilitate the cells migration and proliferation, while the lack of porosity and higher density of 20% PEGDA hinder such migration.

SEM analysis (Fig. 7a–c) confirmed that both hOb and SaOs-2 cells cultured on pddECM/PEGDA hydrogels adhered and spread on the surface after 21 days of incubation. In pddECM/PEGDA hydrogel resin cuts, cell nuclei of hOb and SaOs-2 were found in the whole volume but mostly around pore areas demonstrating thus, cell migration. On the other hand, PEGDA controls only exhibit the presence of cell nuclei in the surfaces and not in the center of the hydrogel (Fig. 7d–l).

4 Discussion

The pddECM was prepared by optimizing previously documented acid extraction and lipid/fat removal procedures to preserve the bioactivity of the extract [20]. In this study, fully demineralized bone particles were obtained within 8 h. This shortened demineralization process might be of great benefit to preserve the ECM collagen structure and potentially its biological activity. Some partially demineralized bone granules remained with treatments of

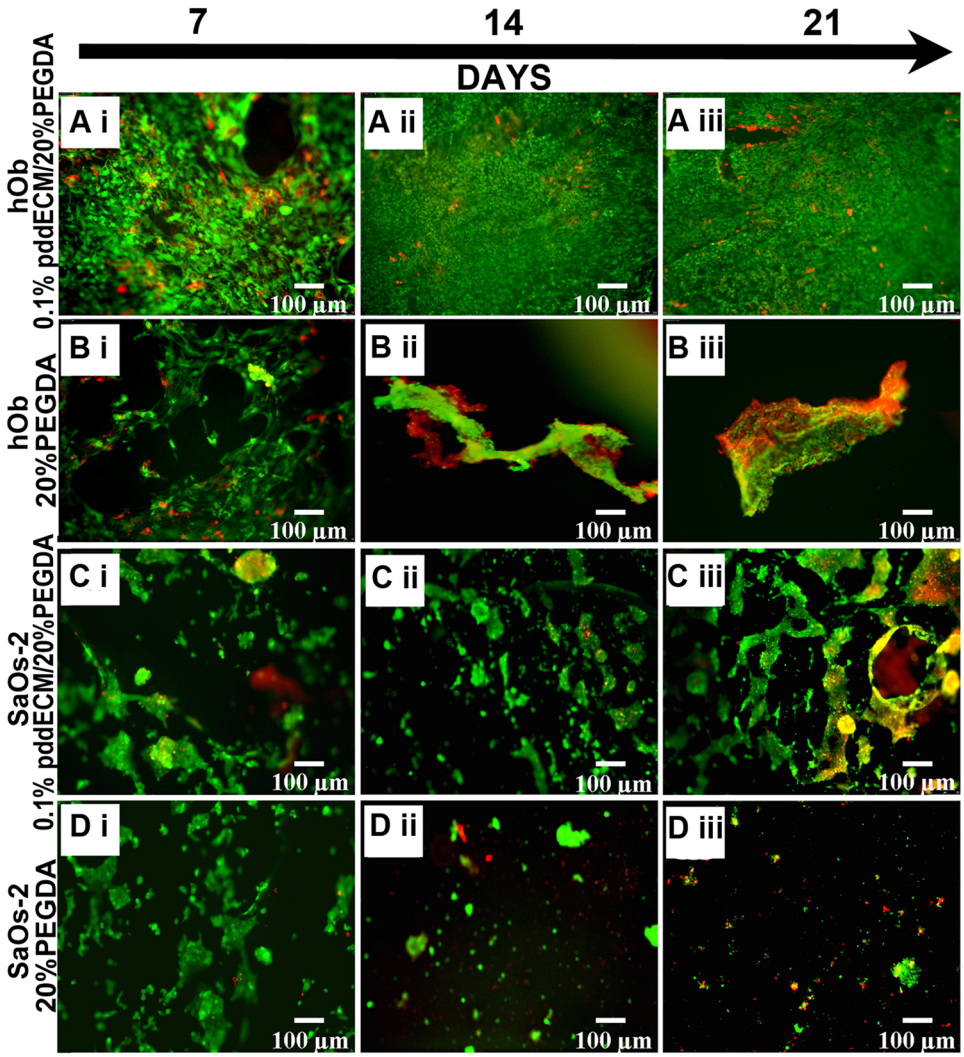

Fig. 5 Live and dead assay of hOb cells on **a** 0.1% w/v pddECM/20% w/v PEGDA hydrogels and **b** 20% PEGDA hydrogels after (i) 7days, (ii) 14 days, (iii) 21 days. Live and dead assay of SaOs-2 cells on **c** 0.1% w/v pddECM/20% w/v PEGDA hydrogels and **d** 20% PEGDA hydrogels after (i) 7days, (ii) 14 days, (iii) 21 days

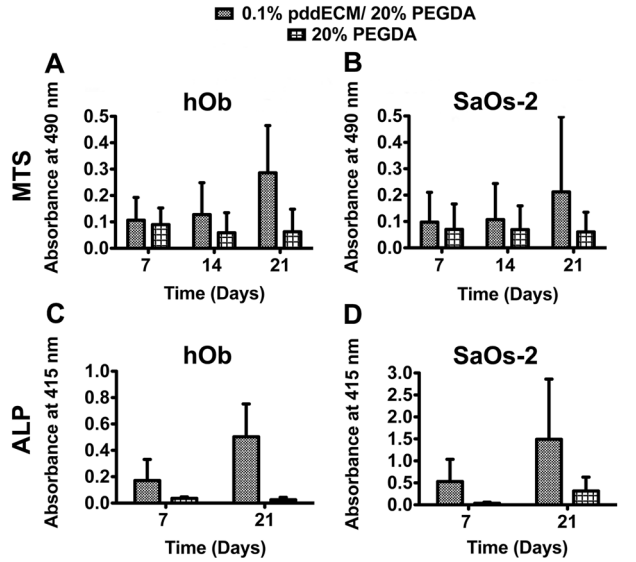

Fig. 6 MTS and ALP assays. **a** hOb cells proliferation assay in 0.1% w/v pddECM/20% w/v PEGDA hydrogels and 20% w/v PEGDA controls hydrogels, absorbance at 490 nm. **b** SaOs-2 cells proliferation assay in 0.1% w/v pddECM /20% w/v PEGDA hydrogels and 20% w/v PEGDA controls hydrogels, absorbance at 490 nm. **c** hOb cells normalized ALP activity in 0.1% w/v pddECM/20% w/v PEGDA hydrogels and 20% w/v PEGDA controls hydrogels, absorbance at 415 nm. **d** SaOs-2 cells normalized ALP activity in 0.1% w/v pddECM/20% w/v PEGDA hydrogels and 20% w/v PEGDA controls hydrogels, absorbance at 415 nm. (*$p < 0.05$; $n = 3$)

less than 8 h of demineralization, and it is hypothesized that prolonged acid extractions could increase the risk of altering the ECM components. Based on the LCMS results, a demineralization of 8 h was sufficient to remove immunoglobulins and fatty acids from the pddECM, which is an essential step to reduce its immunogenicity [38].

Decellularization of the pddECM was achieved by using HCl and extended methanol/chloroform treatment. Tripzinization was not included since the use of this agent may induce partial degradation of the pddECM as demonstrated by electrophoresis (Supplementary Fig. 4) and also suggested in previous studies [37]. Toluidine blue staining and DNA quantification revealed the absence of cells, genomic and mitochondrial dsDNA in the pddECM samples, showing a quantity well bellow the maximum threshold of 50 ng of dsDNA/mg of dry initial weight suggested for implantable biomaterials [20, 37].

The optimization of the buffered-pepsin digestion and further dialysis rendered in an effective technique to achieve a high yield of pddECM minimizing the enzymatic exposure time and therefore, limiting the formation of denatured telopeptides [26, 39]. Growth factors and non-collagenous proteins could not be identified possibly due to acid demineralization, which can deplete small peptides through diffusion from the matrix into the strong acid bath [40].

Sterilization of the pddECM was achieved using high pressurized CO_2 [33]. It was demonstrated that with this unaggressive method was possible to obtain pddECM free of microbial contamination. Alternative procedures using autoclaving, irradiation, ethylene oxide, gas plasma, peracitic acid, and antibiotics should be avoided in order to preserve the functional structure and biological activity of the extract [18, 20, 38, 41], since the collagen amino acids may be irreversibly affected [41]. Previous reports have also shown that the use of strong acids such as HCl, can effectively inactivate viruses such as human rotavirus, vesicular stomatitis [42] and HIV [43]. Moreover, the ability of some chemicals such as ethanol, used during the defatting process, can penetrate the bone within 15 min and provide additional virucidal effects [43].

To facilitate the delivery of the hydrogel blend an injectable form was investigated by loading the pddECM fraction as well as PEGDA separately in a double chamber syringe with a spiral mixer tip. The crosslinking agents were incorporated in a way that the polymers were not affected; thus, preventing premature crosslinking. The injectable formulation demonstrated ease of use and versatility to prevent early polymerization, conditions that are favorable for future clinical applications.

The physicochemical properties were tested in different polymeric concentrations to finally obtain an optimized biomaterial. The gelation kinetics of pddECM/PEGDA hydrogel blends showed that varying the concentration of the polymers has a significant impact on the gelation time as the crosslinking significantly fluctuated from 90 s for the optimized concentration to 220 s for the reduced concentrations. This result may impact the future clinical application by facilitating an adequate in situ onset without losing the material due to patient movement or excessive bleeding during polymerization.

Exposure to blood and inflammatory serum is anticipated in implantable materials; therefore, minimum size variation in the hydrogel scaffold should be achieved. The fabricated pddECM/PEGDA hydrogel demonstrated volumetric stability over time, showing that the optimized concentration generated a SIPN with sufficient internal space to give cells freedom to migrate [44] while preserving control over expansion.

Another major obstacle, is the rapid degradation of natural hydrogels [45]. The space making/keeping effect and timing are paramount requirements of scaffolds in bone tissue regeneration [46]. The injectable pddECM/PEGDA hydrogel blend demonstrated a remarkable controlled degradation preserving the mass structure in approximately 92% for more than 30 days in conditions tailored to match new tissue formation. However, it is hypothesized that macrophages regulating phagocytosis may generate an

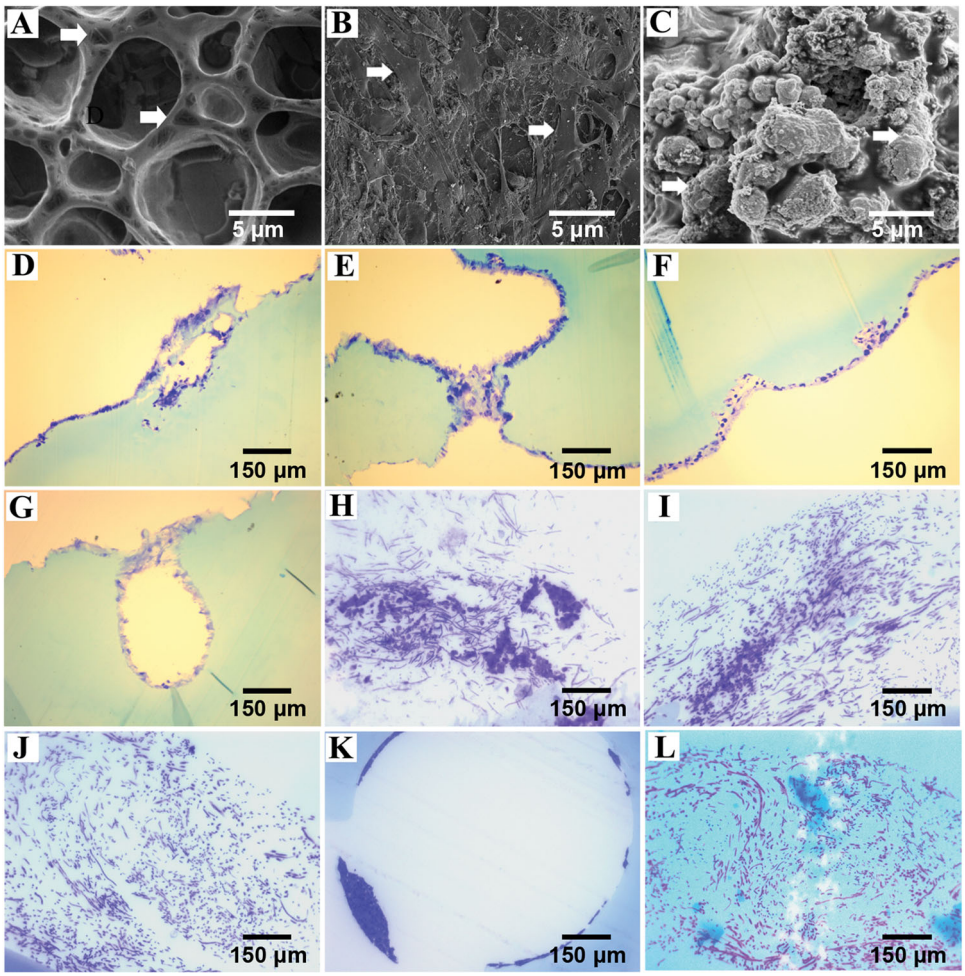

Fig. 7 Scanning electron microscopy of **a** 0.1% w/v pddECM/20% w/v PEGDA hydrogels scaffold ×1000 magnification, white arrows indicating semi-IPN between pddECM and PEGDA polymers. **b** hOb seeded on 0.1% w/v pddECM/20% w/v PEGDA hydrogels ×400 magnification, white arrows indicating hOb flat forms. **c** SaOS-2 seeded on 0.1% w/v pddECM/20% w/v PEGDA hydrogels ×400 magnification, white arrows indicating SaOs-2 flat forms. Resin polymer embedded BMPE/PEGDA and PEGDA hydrogels with Toluidine Blue staining, 3 µm sections indicating **d** Upper area surface of 0.1% w/v pddECM/20% w/v PEGDA hOb seeded hydrogels. **e** Middle area of 0.1% w/v pddECM/20% w/v PEGDA hOb seeded hydrogels. **f** Bottom area surface of 0.1% w/v pddECM/20% w/v PEGDA hOb seeded hydrogels. **g** Middle section pore of 0.1% w/v pddECM/20% w/v PEGDA hOb seeded hydrogels. **h** Upper area surface of 0.1% w/v pddECM/20% w/v PEGDA SaOs-2 seeded hydrogels. **i** Middle area 0.1% w/v pddECM/20% w/v PEGDA SaOs-2 seeded hydrogels. **j** Bottom area surface of 0.1% w/v pddECM/20% w/v PEGDA SaOS-2 seeded hydrogels. **k** Middle section pore of 0.1% w/v pddECM/20% w/v PEGDA SaOs-2 seeded hydrogels. **l** Upper area surface 20% w/v PEGDA SaOs-2 seeded hydrogels as control

inflammatory reaction in the area [47] due to the decreased hydrolytic degradation and the presence of PEGDA.

Nevertheless, the addition of PEGDA to the pddECM fraction substantially improved the mechanics of the extracted matrix by increasing the compression modulus up to 500 kPa. These results are in line with previous findings showing that increasing PEGDA from 10 to 40% can significantly enhance the compression modulus of PLEOF/PEGDA hydrogel blends [30].

Cytocompatibility experiments showed that the cells attached significantly more and spread ubiquitously on the pddECM/PEGDA hydrogel blend compared to PEGDA controls presumably due to the increased number of cellular adhesion sites [48] and the internal spaces. This observation is also in line with previous studies suggesting a critical role of the collagen fraction, which enhance the biological properties of scaffolds [49]. In contrast, PEGDA controls exhibited poor colonization and enzymatic activity possibly due to the lack of intrinsic bioactivity and its compact and dense structure that hinders the cell migration and proliferation capacity.

5 Conclusion

This study demonstrated a biologically optimized process to demineralize, defat and digest bone to obtain ECM collagen

from porcine origin. High pressurized CO_2 sterilization method was used to provide a non-toxic, microbial free and natural extract. The resulting extract containing collagen type I and sub-fractions was blended with PEGDA to generate an SIPN. An injectable formulation was utilized using redox polymerization technique to crosslink the blend after 90 s. The optimized hydrogel blend was shown to be highly cytocompatible, offering a three-dimensional porous microarchitecture that supports and promotes cell proliferation, migration and differentiation. Additionally, favorable physicochemical properties such as high compression modulus, controlled swelling and volume expansion and an appreciable preservation of the polymeric structure suggests a great potential for clinical application in bone defects. The overall composition of the pddECM/PEGDA SIPN hydrogel blend is promising and future in vivo tests to evaluate mineralization rate, inflammatory response as well as bone forming capacity are recommended.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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