Biphasic hydrogels integrating mineralized and anisotropic features for interfacial tissue engineering

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ABSTRACT

The innate graded structural and compositional profile of musculoskeletal tissues interfaces is disrupted and replaced by fibrotic tissue in the context of disease and degeneration. Tissue engineering strategies focused on the restoration of the transitional complexity found in those junctions present special relevance for regenerative medicine. Herein, we developed a gelatin-based multiphasic hydrogel system where sections with distinct composition and microstructure were integrated in a single unit. In each phase, hydroxyapatite (HA) particles or cellulose nanocrystals (CNC) were incorporated into an enzymatically crosslinked gelatin network to mimic bone or tendon tissue, respectively. Stiffer hydrogels were produced with the incorporation of mineralized particles and magnetic alignment of CNC resulted in anisotropic structure formation. The evaluation of the biological commitment with human adipose-derived stem cells (hASCs) towards tendon-to-bone interface, revealed an aligned cell growth and higher synthesis and deposition of tenascin (TNC) in the anisotropic phase, while the activity of the secreted alkaline phosphatase (ALP) and the expression of osteopontin (OPN) were induced in the mineralized phase. These results highlight the potential versatility offered by gelatin-transglutaminase enzyme tandem for the development of strategies that mimic the graded, composite and complex intersections of the connective tissues.

1. Introduction

The interfaces of the musculoskeletal system are complex structures forming connections between dissimilar connective tissues such as fibrous, calcified and cartilaginous materials. These transitional regions are characterized by the presence of gradients in physical, chemical and biological features ¹. Articular cartilage, tendon/ligament enthesis or intervertebral disc are some examples of these transitional interfaces. In particular, structural gradients of extracellular matrix (ECM) composition, collagen molecule alignment and mineralization are observed in the enthesis, the distinct transitional interface region where tendons are anchorage to bones ².

These specialized complex regions present poor ability to promote the restorative processes upon injury or in diseases characterized by degeneration progress. In these cases, a fibrotic scar tissue with considerably different structure and composition from their native graded state is developed, often failing to fulfill their crucial mechanical functions ³. In this scenario, interfacial tissue engineering, which involves interdisciplinary efforts for the development of substitutes that recapitulate the particular complexity present in these regions, is considered an attractive alternative to conventional reparative autograft or allograft transplantation ⁴. The design, development and optimization of single unit constructs with spatially varied biochemical and structural cues are the main objectives of these approaches. For that purpose, several processing techniques have emerged in the last years, and hydrogels systems in particular have showed great progress in this field ⁵. These highly hydrated systems produced from a broad range of hydrophilic polymers simulate the extracellular matrix (ECM) of the tissues ⁶. Enzymatically crosslinkable hydrogels have gained interest since they can be formed under mild conditions. This manufacturing strategy avoids some drawbacks related with other crosslinking strategies

such as the potential cytotoxic effects induced by systems prepared with photo-initiators or the lack of stability associated to physically crosslinked hydrogels ⁷. Peroxidases ⁸, transglutaminases ⁹ and tyrosinases ¹⁰, among other enzymes, have recently been proposed for the production of hydrogels intended for use in regenerative medicine.

Although disparate polymers can be enzymatically crosslinked to produce hydrogels, gelatin owns unique properties that make it an exceptional biomaterial candidate. This natural origin, biodegradable and thermoresponsive polymer presents optimal physical-chemical and biological characteristics for biomedical applications that make it a widely studied material in tissue engineering approaches ^{11, 12}. Numerous nano- and micrometric fillers have been successfully combined with gelatin to produce composites that mimic the musculoskeletal tissues. For example, biocompatible stiff osteoconductive gelatin-hydroxyapatite (HA) structures were developed by cryogelation technique for bone regeneration purposes ^{13, 14}. On the other hand, the mimicry of the anisotropic structure that characterizes tissues such as cartilage, tendons or ligaments is extremely important since their ordered ECM architecture and cellular organization play an essential role in the biomechanical and biological functions of these tissues ¹⁵. Interestingly, different strategies using a range of polymers, including gelatin, as the main matrix material and manipulating the orientations and aggregation of nanoparticles by external magnetic, electric or acoustic fields are being explored to create hydrogels with bioinspired ordered structures ¹⁶. In this context, rod-shaped cellulose nanocrystals (CNC), extracted from the crystalline regions of the cellulose microfibrils, have received particular attention. These nanoparticles own unique properties for tissue engineering applications such as excellent mechanical behavior, biocompatibility and sensibility to relative orientation under magnetic field

¹⁷. Recently, it has been shown that despite the diamagnetic character of the CNC, it is possible to manipulate their orientation within a hydrogel matrix using moderate magnetic fields ¹⁸. In connection with these findings, we previously developed injectable anisotropic gelatin hydrogels loaded with CNC decorated with magnetic nanoparticles under exposure to uniform magnetic fields of low strength ⁹. Both studies have demonstrated that the anisotropic microstructure of the resulting hydrogels can induce the directional organization of seeded and encapsulated cells.

However, tissue engineering strategies that recapitulate in a single integrated construct the dissimilar composition, architecture and cellular organization found in the musculoskeletal tissue interfaces, have not been explored in depth. The development of such graded multiphasic structures that allows the encapsulation of cells seems particularly challenging. In fact, current technologies to fabricate the pre-formed scaffolds often involve too harsh conditions for cell encapsulation and the assessment of *in vitro* biological performance is limited to cell seeding on the prefabricated scaffolds ¹⁹. For instance, extreme pressure, non-physiological salt concentration and the use of organic solvents are some of those situations. Promisingly, the development of hydrogels under suitable conditions for three-dimensional (3D) cell encapsulation and culture provides more realistic biochemical and biomechanical microenvironments ²⁰.

Here, we propose a novel methodology to develop a hierarchically structured hydrogel where bone and tendon tissue related biochemical and structural cues are gradually integrated in a single construct. Our hypothesis is that microbial transglutaminase will allow the crosslinking of the remaining free reactive groups of independent gelatin hydrogels to integrate dissimilar phases

into a single construct in order to recapitulate the graded composition, architecture and cellular organization found in the native enthesis. For the mineralized phase that mimics the bone tissue, gelatin hydrogels were loaded with osteoinductive HA particles. For the generation of tendon tissue inspired anisotropic hydrogels, CNC were incorporated and exposed to magnetic fields during the crosslinking process. The morphological and mechanical properties of the composite hydrogels were thoroughly evaluated. Their biological performance was assessed by the encapsulation of human adipose-derived stem cells (hASCs) into the systems (mono or biphasic), and cell alignment and the expression of bone and tendon tissue related markers were determined.

2. Experimental section

2.1 Materials

Gelatin from porcine skin (Type A, gel strength 300), microcrystalline cellulose (MCC, Avicel), sulfuric acid 95-97%, phosphate buffered saline (PBS), bovine serum albumin, dialysis tubing cellulose membrane (MWCO 12-14 kDa), p-nitrophenyl phosphate (pNPP) and phalloidin tetramethylrhodamine B isothiocyanate (phalloidin-TRITC) were purchased from Sigma-Aldrich, Portugal. Microbial transglutaminase (mTG) was obtained from Ajinomoto, Japan. HA microparticles were purchased from Fluidinova, Portugal. Neutral buffered formalin 10% (v/v) and Triton X-100 were obtained from ThermoFisher Scientific. Minimum essential medium alpha (α -EMEM), antibiotic/antimycotic solution (A/A), fetal bovine serum (FBS) and TrypLE Express with phenol red were purchased from Life Technologies. Normal horse serum 2.5% was obtained from Vector Laboratories; 4,6-diamidino-2-phenylindole dilactate (DAPI) from

Biotium, USA; and silicone squared molds (12 well chamber removable) from Ibidi, Germany. Alexafluor 488 donkey anti-rabbit and anti-mouse were purchased from Invitrogen.

2.2 Synthesis and characterization of cellulose nanocrystals (CNC)

The synthesis of CNC was achieved hydrolyzing MCC with sulfuric acid according to the protocol previously described by Bondeson et al, with minor adaptations ²¹. In brief, MCC/water suspension was prepared by mixing 42 g of MCC powder with 189 mL of deionized water and mechanically stirred at 500 rpm during 10 minutes, immersed in an ice bath to avoid overheating. Next, 188.3 mL of concentrated sulfuric acid 96% was added dropwise up to a final concentration of 64% w/w. The acid hydrolysis was performed heating the suspension to 44 °C and maintaining the vigorous stirring at 500 rpm for 2 hours. After that, the reaction was quenched by diluting the suspension with distilled cold water (5 times the initial volume) and the solution was left to decant at 4 °C for 2 hours. After discarding the acidic supernatant, the remaining suspension was centrifuged for 10 minutes, at 9000 rpm and 5 °C. The supernatant resulting after the centrifugation process was discarded and replaced by ultrapure water. The suspension was centrifuged until the supernatant became turbid. The resulting final suspension containing CNC was widely dialyzed against deionized water, using cellulose membranes (MWCO: 12-14 kDa) until neutral pH was reached. Consequently, the slurry content was poured into beakers and three cycles of 10 minutes sonication (VCX-130PB-220, Sonics) was applied to completely disperse the aggregates of nanocrystals. The ultrasound probe was set at 60% of amplitude output and an ice cooling bath was used to avoid overheating during the process. Finally, the cloudy suspension was centrifuged for 10 minutes at 9000 rpm and 5 °C to remove

minor MCC residues that have not been effectively disintegrated into colloidal CNC, and the final supernatant containing CNC was stored at 4 °C until further use.

Atomic force microscopy (AFM Dimension Icon, Bruker, USA) was used to characterize the produced CNC, following the protocol described elsewhere ^{22, 23}. Briefly, one drop of 0.0015% w/v CNC suspension in ultrapure water was placed on a freshly cleaved mica disk and after removing the excess liquid, it was dried overnight. The AFM was used in PeakForce Tapping (ScanAsyst) in air mode with a MultiMode AFM connected to a NanoScope V controller (Veeco, USA). The scans were collected using a silicon nitride AFM cantilever (ScanAsyst-Air, Bruker) with a spring constant of 0.4 N.m⁻¹ and frequency of 70 kHz. CNC dimensions were calculated as previously described and ranged from 4 – 7 nm in height and 70 – 400 nm in length ^{22, 23}.

2.3 Development of enzymatically crosslinked hydrogels

Enzymatically crosslinked gelatin hydrogels were prepared using microbial transglutaminase (mTG) enzyme. Nanocomposite hydrogels and mineralized hydrogels were developed by incorporating CNC or HA microparticles in the formulation, respectively. Gelatin from porcine skin was dissolved in 0.1X PBS solution at 10 % (w/v) under constant stirring at 60 °C for 2 hours and then cooled to 37 °C. The mTG solution was prepared at RT, dissolving the enzyme powder (100 U/g) at a concentration of 20% (w/v) in PBS. CNC suspension at different concentration (1 and 2 % (w/v)) was dissolved in ultrapure water, pH neutralized with 0.2 M NaOH and dispersed with ultrasonic processor (40% Amplitude output, 20 seconds, 3 cycles) prior to mixing with the polymer solution. Gelatin solution was mixed in a 1:1 (v/v) ratio with

the CNC suspension and the required amount of mTG solution was added to obtain 10 U/g gelatin enzymatic activity. To prepare the final isotropic hydrogels, the resulting solutions were thoroughly mixed by pipetting up and down, dispensed into silicone-squared molds (6x6x4 mm) and kept at 37 °C in humid atmosphere for 1 hour to allow crosslinking. Instead, for the preparation of anisotropic hydrogels the molds containing the hydrogels solutions were kept under the influence of uniform magnetic fields in a custom-made magnetic system, consisting of two neodymium permanent magnets N52. Two different magnetic force levels were employed by adjusting the distance between the magnets, resulting in magnetic field strength of 200 mT and 400 mT. A gaussmeter (Hirst Magnetic Instruments) was used to determine the strength of the applied magnetic field. For the preparation of mineralized hydrogels, HA micrometric sized spherical particles (Particle size = $5.0 \pm 1.0 \mu\text{m}$, Fluidinova, Portugal) were incorporated into the gelatin solution. Both the gelatin and HA particles were dissolved at the same time, using the conditions described above. Similarly, the necessary volume of enzyme was added to the mixture, and the crosslinking process was completed at 37 °C for 1 hour. Moreover, biphasic hydrogels with differentiated sections were developed by casting the pre-solution of a new hydrogel on the top of the crosslinked hydrogel. Thus, mineralized isotropic phase and nanocomposite anisotropic phase were overlapped. The composition and the preparation conditions of each hydrogel are summarized in the table of Figure 1D.

2.4 Characterization of developed hydrogels

2.4.1 Scanning electron microscopy (SEM)

The microstructural morphology and organization of nanocomposite and mineralized hydrogels was analyzed by high-resolution SEM (JSM-6010LV, JEOL, Japan). The samples

were freeze-dried first, cooled and fractured in liquid nitrogen then, and sputter-coated with platinum after (Cressington). The image acquisition was performed with an acceleration voltage of 10 kV. ImageJ software was used for the analysis of the images. SEM images were converted to binary and erode or dilate functions were applied. These adjusted files were used for the determination of shape description parameters via the analyze particles function. At least 50 pores per formulation were assessed. For the analysis of the organization of the pores, directionality determination was performed using the Fourier components method.

2.4.2 Mechanical characterization

The compressive mechanical behavior of the developed hydrogels were analyzed using an universal mechanical testing equipment (5543K2942, 5543, Instron) equipped with a 1 kN load cell, under a compression rate of 1 mm/min. The samples were prepared in molds with a uniform cylindrical shape (6x6x5 mm). Prior to the test, the size of the hydrogels was measured using a digital caliper. The Young's compressive modulus was determined from the slope of the stress-strain curve in the 10-25% strain linear region. Same experimental settings were applied to evaluate at least five different samples per each composition.

2.4.3 Rheological analysis

The rheological properties of the nanocomposite hydrogels were measured using a rheometer AR1000 (TA instruments, New Castle, USA) and a 40 mm diameter plate test geometry was used. 1 mL of the solutions was directly dispensed while in liquid state on the bottom Peltier plate, previously heated at 37 °C. The gap was adjusted to 0.5 mm and a thin film of paraffin oil was used to avoid the water evaporation from the samples. Time-sweep tests were conducted at a

frequency of 0.5 Hz and shear strain of 0.5%, monitoring values of storage (G') and loss moduli (G'') for 75 minutes.

Isotropic nanocomposite cylindrical hydrogels were prepared for the analysis of bulk viscoelastic properties. For that, oscillatory frequency sweep tests (0.01 - 10 Hz) were carried out at 37 °C, applying an initial pre-adjusted normal force of 1 N and a constant strain amplitude of 0.5%. Three replications were performed for each formulation, representing the average of those values as the final result.

2.4.4 Polarized optical microscopy

Polarized optical microscopy was used to evaluate the anisotropic hydrogel network and the time required to achieve the alignment of the CNC under magnetic field. Thin hydrogels were produced by deposition of 100 μ L of the hydrogel precursors in the molds mentioned above. The formulations were physically crosslinked at each time point by cooling the system with an ice bath. Images were acquired using an optical microscope equipped with a digital camera. Samples were placed between crossed polarizers and 10x magnification was used for all images.

2.4.5 Micro-computed tomography (μ -CT)

The biphasic hydrogels were assessed by high-resolution μ -CT analysis (Skyscan 1272, Skyscan, Belgium). Two dimensional projections with a pixel resolution of 4.96 μ m were acquired over a rotation range of 360° and with a rotation step of 0.4°, by cone-beam acquisition after irradiating the specimens with penetrative X-rays using a source voltage of 60 kV and a

current of 166 μ A. The 3D image was reconstructed using the NRecon software (version: 1.7.1.0).

2.5 Biological performance of hydrogels

2.5.1 Cell culture

Human adipose tissue-derived stem cells (hASCs) were used to evaluate the biological behavior of the developed hydrogels. hASCs were isolated from lipoaspirate samples obtained from Hospital da Prelada (Porto, Portugal). All the procedures were approved by the Ethical Committee of the Hospital da Prelada and University of Minho. The hASCs isolation and stemness characterization were performed following a protocol described elsewhere ²⁴. Briefly, the tissue samples were thoroughly washed with PBS and digested with 0.05% collagenase Type I A (Sigma-Aldrich) in PBS for 60 min at 37 °C under gentle stirring. Then, cells were centrifuged and the cell pellet was resuspended in basal cell culture medium (α -MEM supplemented with 10% (v/v) FBS and 1% (v/v) A/A) and seeded in culture flasks. After 24 hours of incubation at 37 °C and 5% CO₂, the adherent cells were washed and their stemness was evaluated through flow cytometry for the expression of mesenchymal stem cell markers (CD45, CD105 and CD90), as shown previously ²⁵. For all experiments, hASCs were maintained in culture with basal medium and used at passage 3-6.

2.5.2 Cell encapsulation in 3D hydrogels

The hASCs cells were encapsulated in the 3D hydrogels. The gelatin 10% (w/v) solution prepared in 0.1X PBS was supplemented with 25 mM of sucrose, with the aim of getting a suitable environment for cellular growth, adjusting the osmotic pressure for it. This solution and

the dissolution of mTG enzyme were filtered using 0.22 μm filters. On the other hand, the suspension of nanocrystals was first dispersed by ultrasonic processor (40% amplitude, 30 seconds, 3 cycles) and placed under UV radiation for 30 min after. 10^6 cells were resuspended in 500 μL gelatin solution and the cellular suspension in gelatin, the enzyme solution and the CNC suspension were mixed as described before. The volume necessary to achieve 1% A/A in the final formulation was added. The isotropic and anisotropic hydrogels were prepared as previously described by adding 100 μL of the mixture in the silicon squared molds mentioned above. The cell containing hydrogels were kept at 37 °C during 60 min for the crosslinking phase. After that, the hydrogels were removed from the molds and placed in 24 well plates. 1 mL of basal medium was added to each well, changing it every 2 days over the course of the cellular assays.

2.5.3 Cell organization evaluation

Cell nuclei and cytoskeleton actin filaments were stained and confocal laser scanning microscope (Leica TCS SP8, Mycosystems, Wetzlar, Germany) was used to evaluate the cellular morphology and organization. At each time point, the hydrogels with encapsulated hASCs were fixed with neutral buffered formalin 10% (v/v) at RT for 30 min. After washing the samples two times with PBS, a solution of Triton X-100 at 0.2% (v/v) in PBS containing DAPI (1:500, v/v) and phalloidin-TRITC (1:200, v/v) was used for the staining, maintaining 1 hour protected from light under gentle shaking (130 rpm). A final washing step with PBS was conducted to eliminate the excess dye. Images from the samples were acquired through confocal microscopy. Nuclei aspect ratio was evaluated measuring at least 50 nuclei for each condition using Image J software. The nuclei aspect ratio was estimated by dividing the length by the

width, achieving ratios > 1 for elongated nuclei. For cytoskeleton organization evaluation, directionality plugin with Fourier components method from Image J software was applied to the images corresponding to actin filaments. The histograms were fit to the Gaussian distribution function using Origin software. As a result, the full width at half maximum (FWHM) was determined for each group.

2.5.4 Immunofluorescence staining for bone and tendon-related markers

Immunostaining for specific osteogenic and tenogenic markers was conducted after 6 and/or 21 days of culture. Before the staining, hydrogels were washed with PBS and fixed in formalin 10% (v/v). For cell permeabilization Triton X-100 at 0.1% (v/v) in PBS was used for 20 minutes under gentle agitation. After rinsing the samples three times with PBS, normal horse serum 2.5% was added to block unspecific reactions and maintained for 90 minutes at RT. Then, hydrogels were incubated with primary antibodies against osteopontin (OPN) (rabbit anti-osteopontin antibody, 1:1000, Abcam ab8448) and tenascin (TNC) (mouse anti-tenascin-C antibody, MAI-26779, ThermoFisher Scientific, 1:3000). The samples were incubated overnight at 4 °C under gentle shaking. After removing the antibody solution, a washing step with H₂O₂ at 0.3% (v/v) for 15 minutes was carried out. Thereafter, the samples were incubated with the corresponding secondary antibody labeled with AlexaFluor 488 (1:200), for 2 hours at RT protected from light under mild agitation. All the antibodies were prepared in BSA 0.1% (w/v) in PBS. Finally, once the samples were washed with PBS, nuclei and cytoskeleton were stained with DAPI (1:500 in PBS) and phalloidin-TRITC (1:200 in PBS), respectively, at RT for 1 hour. After a final washing, the samples were kept in PBS at 4 °C until the imaging. The immunostained hydrogels were analyzed with the confocal microscope. The maximum projections for Z stacks confocal

images were obtained and the evaluation of specific signal was normalized per nuclei area. The pixels corresponding to background signal were discarded applying a threshold, and the area of immunolabeled pixels was divided by the area corresponding to blue nuclei pixels. In the case of TNC staining, the directionality analysis was also completed following the same protocol described for the cytoskeleton organization evaluation.

2.5.5 Alkaline phosphatase (ALP) activity determination

The secretory form of ALP from hASCs was monitored during 3 weeks. The activity was evaluated by determining the hydrolysis of p-nitrophenyl phosphate by ALP at pH 9.3. A standard curve with calf intestinal ALP was prepared and 100 μ L of pNPP at 0.2% (w/v) was added to each sample. The reaction was stopped with 50 μ L of NaOH 3 M and absorbance measured at 405 nm.

2.6 Data analysis and statistics

The statistical analysis of the data was completed using GraphPad PRISM (7.0) software. For normally distributed data, Student's t-test or one-way ANOVA were applied for differences between two groups or multiple comparisons, respectively. Tukey post-hoc test was applied for multiple comparisons. For non-normally distributed data, Mann-Whitney nonparametric analysis or Kruskal-Wallis test with Dunn's multiple comparisons test were applied. In all cases, p values < 0.05 were considered as significant, represented by symbols described in the graphs. Data are presented as mean $\pm$ standard deviation. For all experiments, at least three independent samples per each condition were assessed.

3. Results and discussion

3.1 Development of enzymatically crosslinked hydrogels

Gelatin, a natural origin polymer, was enzymatically crosslinked by mTG enzyme and specific micro and nanometric particulate fillers were incorporated into the constructs, in order to obtain hydrogels with specific tissue mimetic characteristics (Figure 1A). In this regard, HA spherical 5 μm microparticles were selected to provide osteoinductive features to the formulation (Figure 1C) ^{26, 27}. HA is the main inorganic component of the mineral phase of native bone tissue. Furthermore, the capability of this material to support osteogenic differentiation of mesenchymal stem cells from different sources, such as adipose tissue ²⁸ or bone-marrow ^{29, 30} is well established. On the other hand, rod-shaped CNC were incorporated into the formulations to act as both reinforcement nanomaterial and as a guide for the development of anisotropy under magnetic stimulation (Figure 1C). These nanoparticles are broadly used in tissue engineering approaches to achieve added functionalities to hydrogels systems, such as electrical or magnetic responsiveness ³¹. Magnetic fields locally induce a perpendicular alignment of CNC nanorods with respect to the field direction due to their negative diamagnetic susceptibility ($-0.95 \times 10^{-6} \text{ m}^3 \text{ kg}^{-1}$) and morphological anisotropy ^{18,32}. The CNC anisotropic diamagnetic susceptibility stem from the magnetic dipole of individual C–O, C–H, and O–H bonds and their relative orientations in the cellulose polymer chain ¹⁸.

The enzyme applied for the fabrication of these systems catalyzes the reaction between ϵ -amino group of lysine and γ -carboxamide group of glutamyl residues, giving as a result stable intra and intermolecular isopeptidic bonds in physiological conditions ³³. This enzymatic strategy of getting covalently crosslinked gelatin hydrogels has been previously proposed by other

authors to design biocompatible *in situ* gelling injectable formulations without remarkable immunogenic or cytotoxic aspects ³⁴. In addition, this enthralling approach presents an exceptional versatility to combine diverse compositional hydrogels. In this work, unique structures composed of differentiated phases (mineralized phase vs anisotropic phase) with well-integrated interface have been successfully developed (Figure 1B, Video S1 Supporting information). Biphasic or even triphasic systems (Figure S1) were produced by stacking a new gelatin solution on the top of previously crosslinked hydrogel. The catalytic activity remaining on the surface of the preformed structure allows the integration of the new gelatin network.

Figure 1. Schematic representation of developed gelatin-based hydrogels. (A) Enzymatically crosslinked gelatin networks reinforced with HA or CNC particles, achieving anisotropy under magnetic field exposure. (B) Digital image of produced biphasic hydrogel with well-differentiated phases. (C) SEM image of spherical hydroxyapatite microparticles (scale bar= 20 μ m) and AFM image of cellulose nanocrystals (scale bar = 500 nm). (D) Summary table of the

composition and preparation conditions of the developed hydrogels. A sample ID given to each system is applied throughout the entire manuscript.

3.2 Characterization of gelatin nanocomposite constructs

Morphological characterization was completed by microstructure analysis of hydrogels representative SEM images. Freeze-dried isotropic and anisotropic hydrogels with 0.5% and 1% (w/v) CNC were cross-sectioned in order to evaluate the internal organization. As shown in the images depicted in Figure 2A, porous structures were obtained in all cases. Randomly oriented pores without any organization were visualized in the isotropic samples prepared in the absence of magnetic field. However, more organized pores arrangement was observed in hydrogels exposed to magnetic alignment during the crosslinking phase. These structural differences were proved by directionality analysis of the images (Figure 2B). The structural alignment observed in the anisotropic systems, resulted in a pointed frequency peak at a particular angle of orientation, without any remarkable difference between both concentrations. Conversely, multimodal flat distribution was obtained for both isotropic hydrogels. Regarding the morphology of the pores, lower data scattering of the aspect ratio was achieved for anisotropic systems (Figure 2C), demonstrating higher shape regularity in the anisotropic samples. In relation to the pore size, larger pores were formed with structural alignment, which may be due to the spatial reorganization of the gelatin strands. Nonetheless, higher concentration on CNC resulted in smaller pores (Figure 2C). However, the pore size of all these formulations (6-22 μm) falls within the range to allow the diffusion of oxygen, nutrients and metabolic products to cells ³⁵.

Alignment of pure CNC can be induced at relatively weak magnetic fields (>0.56 T) above the critical nanoparticle concentration required for nematic liquid crystals formation ³⁶. However, it

has also been recently demonstrated that adsorption of polymers on CNC surface increases its effective volume fraction, leading to increased nematic structuring and therefore increased susceptibility to magnetic alignment^{37,38}. These effects are hypothesized to enable the magnetic alignment of CNC at weaker magnetic fields and below the critical concentration required for nematic ordering, such as those explored in this work. To further evaluate the anisotropy of the proposed systems upon exposure to 200 and 400 mT magnetic fields, polarized optical microscopy was used. Continuous multi-chromatic transmission images were observed in the case of gelatin nanocomposite gels produced without external magnetic exposure (IsoGEL0.5 and IsoGEL1). This result shows the development of multi-domain structures with heterogeneous birefringence due to the random organization. In contrast, a chromatic transition from multichromatic to monochromatic is displayed in few minutes when the systems are exposed to a low uniform magnetic field (200 mT) (Figure 2D). Monochromatic images were visualized after 15 minutes of magnetic exposure for the hydrogels containing both concentrations of CNC tested. This result reflects the existence of a mono-domain structure that presents homogenous birefringence covering the entire region. However, this effect was achieved faster with the hydrogels containing 1% (w/v) CNC since images of a single color were observed after 10 minutes. As it can be seen in the images depicted in Supporting Figure 2, by increasing the applied magnetic field strength to 400 mT, faster CNC alignment is achieved, getting mono-domain structures at 10 minutes in both cases. These results are in accordance with previously conducted studies on the analysis of the magnetic alignment of CNC suspensions, where fast initial ordering within minutes in response to increasing magnetic field strengths was observed³⁶. Similarly, Omidinia-Anarkoli et al. concluded that the orientation time of magneto-responsive

poly(lactide-co-glicolide) microfibers containing superparamagnetic iron oxide nanoparticles decreases with increasing the magnetic field strength ³⁹.

Figure 2. Morphological characterization and optical properties assessment of hydrogels containing CNC. (A) Scanning electron micrographs of the freeze-dried hydrogels (scale bars = 50 μ m). (B) Structural directionality evaluation of isotropic and anisotropic hydrogels. (C) Analysis of descriptive parameters of the pores; the aspect ratio and pore size. Statistical differences: **** $p < 0.0001$. (D) Polarized optical microscopy images taken during the crosslinking phase with or without 200 mT magnetic field exposure (scale bars = 1 mm).

To study the impact of the CNC incorporation on the rheological and mechanical properties of the developed hydrogels, we performed time-sweep tests to determine the gelling point and frequency-sweep to assess the bulk viscoelastic properties. The incorporation of the CNC within the gelatin network enzymatically crosslinked with the mTG enzyme proved to have no influence on the gelation time. In all cases, the formulations were fully crosslinked and the 3D systems were structured between 17 and 20 minutes (Figure 3A). This data shows that the gelling time for all the formulations is enough to allow the alignment of CNC (Figure 2D). This time-range is similar to other *in situ* gelling injectable formulations proposed for tissue engineering applications^{40, 41, 41}. With respect to isotropic hydrogels bulk viscoelastic properties (Figure 3B), data exhibited an increase in the elastic modulus (G') value proportional to the concentration of nanoparticles used. Hence, gelatin hydrogel without reinforcing nanofiller showed G' of 524 ± 12 Pa, while up to 4-fold stiffer isotropic hydrogel were developed with 1% (w/v) CNC incorporation (2450 ± 209 Pa). These results demonstrate the convenience of these nanoparticles as reinforcing elements, due to the good dispersion within the matrix. Finally, to prove the anisotropic mechanical properties resulting from the CNC alignment after exposure to different strength magnetic fields, mechanical compression tests perpendicular to the alignment of the CNC were performed. As can be seen in Figure 3C, the higher the proportion of CNC in the hydrogel, the stiffer structures were obtained. The hydrogels prepared with 0.5% (w/v) CNC in the absence of magnetic field showed Young modulus of 17.36 ± 4.12 kPa, while the formulation comprised of 1% (w/v) and produced under the same conditions presented compressive modulus of 27.31 ± 6.83 kPa. Besides, the exposure to a magnetic field during the gelation process increased the compressive properties, confirming the presence of anisotropic microstructures in these hydrogels. Reasonably, the existence of strictly arranged planes of aligned rigid CNC

perpendicularly oriented to the course of compression might enhance the resistance to compression of the hydrogel³⁸. In fact, the highest Young Modulus was achieved by AnisoGEL1_400 sample (38.37 ± 4.85 kPa).

Figure 3. Mechanical characterization hydrogels containing CNC. (A) Rheological time-sweep curves of isotropic hydrogels with 0, 0.5 and 1% (w/v) CNC. (B) Elastic modulus (G') from frequency-sweep of isotropic systems. (C) Young Modulus determined from strain-stress curves linear region under compression force. Statistical differences: * $p < 0.05$ compared with isotropic hydrogel with the same concentration of CNC; # $p < 0.05$ and ## $p < 0.01$ compared with the hydrogels exposed under the same magnetic field.

3.3 Biological performance of CNC-loaded hydrogels

hASCs were used to evaluate the biological performance of gelatin nanocomposite hydrogels. First, a preliminary study was completed to determine the biocompatibility of the enzymatically crosslinked nanocomposite systems. For that, the viability of cells encapsulated into the isotropic hydrogels was evaluated by live/dead staining. As can be seen in Figure S3, the cells cultured in the 3D systems for three days showed high viability, without significant differences between groups. These results demonstrate that the designed nanocomposite biomaterials are not cytotoxic and that they are suitable for cellular growth support.

Next, the effect of hydrogel alignment on cytoskeleton organization was determined. Constructs containing 0.5 and 1% (w/v) CNC were exposed to uniform magnetic fields of 200 and 400 mT during the crosslinking phase. Isotropic hydrogels containing the same amount of CNC but crosslinked in absence of external magnetic stimulus were considered as controls. The cellular orientation was observed under confocal microscopy after 3, 7, and 21 days of culture. After 3 days of culture, hASCs encapsulated within the anisotropic hydrogels displayed a spindle-shape morphology and they were more spread than in control hydrogels (Figure 4A). Besides, it was possible to appreciate a certain cell orientation preference within the anisotropic hydrogels, which was confirmed by cytoskeleton directionality analysis. While multimodal flat distribution was shown by both isotropic hydrogels, unimodal narrow distribution tendency could be deduced from all anisotropic hydrogels histograms. This data was further confirmed from the Gaussian distribution fitting, since FWHM values of anisotropic hydrogels were significantly lower (FWHM $\sim 40^\circ$) compared with the analogue isotropic samples (FWHM = $121.3 \pm 34.65^\circ$ for IsoGEL0.5 and $148.3 \pm 44.81^\circ$ for IsoGEL1). Indeed, the size, geometry or even the spatial organization of nanotopographical cues could have an influence on stem cells

adhesion, migration, proliferation, morphology and differentiation fate, essentially through coordinated mechanotransduction systems. Several molecular pathways such as FAK/Src, Rhoa/ROCK or LINC complex are responsible for the transference of stimulus from ECM to the nucleus ⁴².

After 7 days of culture, the cell density was notoriously higher in all the formulations, which suggests a suitable cell proliferation thereof. hASCs encapsulated in gelatin-based nanocomposite hydrogels were overall fusiform and they were completely spread through the 3D environment (Figure 4B). In the case of cells embedded in isotropic systems, random organization without any preferential orientation was observed. Otherwise, in the case of anisotropic hydrogels, the cellular alignment observed after 3 days of culture was maintained also after 1 week. This alignment was confirmed by directionality analysis and sharp peaks at specific angles were noticed for anisotropic constructs, getting values of FWHM around 60°. Considering that no magnetic field was applied during the entire culture period, the cellular alignment observed after 7 days was an outcome of the intrinsic anisotropy of the hydrogels, as suggested in previous studies performed with anisotropic systems ^{9,43,43}. Regarding the influence of the magnetic field strength used to produce anisotropic hydrogels on cell organization, no significant differences were noticed between 200 or 400 mT exposed hydrogels. These results suggest that although the higher magnetic field used in this study leads to increased hydrogel physical anisotropy, it is not different enough to impact 3D cell organization. Therefore, considering the overall physical-chemical properties and the influence over cell organization, AnisoGEL1_400 formulation was selected for further cellular studies.

Figure 4: Evaluation of encapsulated hASCs cytoskeleton alignment in 3D hydrogels. Confocal fluorescence images of cellular cytoskeleton (F-actin, red) (scale bar = 200 μm), the directionality histograms of actin filaments distribution and the full width half medium (FWHM)

of the histograms determined by Gaussian fitting of the curves after 3 days (A) and 1 week (B) of culture. Statistically significant differences between isotropic and anisotropic hydrogels with the same concentration of CNC are shown as * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$.

Selected formulations were maintained until 21 days of cell culture (IsoGEL1 and AnisoGEL1_400) and morphological and biochemical cues related to anisotropic tissues were evaluated. Thus, nuclei aspect ratio, directionality of cell distribution and tendon-related ECM protein TNC secretion⁴⁴ were assessed by confocal immunofluorescence (Figure 5A). The nuclei of the cells encapsulated within magnetically assisted platforms showed a tendency to elongate, obtaining higher values in nuclei aspect ratio (Figure 5B). Accordingly, the alignment of the cytoskeleton was much more pronounced for these samples, presenting a main orientation against the random distribution in the isotropic systems (Figure 5C). Finally, the amount of TNC expression per nuclei was greater in the anisotropic hydrogels (Figure 5E) and the deposition of this glycoprotein was displayed in a much more fibrillar and organized way, being consistent with the cell alignment orientation (Figure 5D). This oriented structural arrangement of secreted TNC has been previously described. For example, controlled parallel spatial deposition of ECM proteins to aligned polycaprolactone/silk fibroin nanofibers from seeded dermal fibroblasts was concluded in rabbit Achilles tendon defect model⁴⁵.

All together, the data achieved with AnisoGEL1_400 hydrogel suggests its potential to simulate anisotropic environment found in some native tissues, such as in tendon tissue.

Figure 5. Biological characterization of the nanocomposite 3D hydrogels using encapsulated hASCs after 21 days of culture. (A) Confocal images of immunolabeled samples against tenascin (TNC; green), cells nuclei (blue) and cytoskeleton (red) (Scale bars = 200 μm). (B) Nuclei aspect ratio of cells cultured in isotropic and anisotropic (400 mT) hydrogels reinforced with 1% (w/v) CNC. Evaluation of the cytoskeleton (C) and the TNC deposition (D) directionality by the encapsulated cells. (E) Quantification of tendon-related marker TNC expression normalized with nuclei area. Statistical significance: **** $p < 0.0001$.

3.4 Mineralized hydrogels: characterization and evaluation of bone commitment by hASCs

HA particles were added into the enzymatically crosslinked gelatin hydrogels, in order to get mineralized hydrogels to better mimic the bone phase present in the enthesis. The osteoinductive

microparticles were broadly distributed and well-dispersed around the entire hydrogel network, without appreciable aggregation tendency. The evaluation of the freeze-dried samples by SEM allowed assessing the disposition of the particles into the 3D system. These images are depicted in the Figure 6A, and it was confirmed that the mineralized particles were wrapped by the gelatin network. Therefore, constructs displaying porous microstructures with mineralized cues distributed throughout the system were developed.

Regarding the compressive properties of the mineralized hydrogels, significantly higher Young Modulus (43.28 ± 10.14 kPa) was achieved in comparison to hydrogels prepared without HA microparticles (Figure 6B). This reinforcing effect might be interesting for those constructs aimed at stiffer tissues applications. In fact, this value is in the same range of osteoid stiffness, the bone precursor crosslinked collagen secreted by osteoblasts ⁴⁶.

For the biological performance evaluation of mineralized hydrogels, hASCs were encapsulated in 3D constructs and bone-related markers were analyzed, comparing the data with unloaded gelatin hydrogels. The activity of ALP secreted by the cells (Figure 6C) was used to determine the potential of the system to guide osteogenic differentiation of hASCs ^{29, 29, 47}. Whereas the activity of ALP secreted by cells encapsulated within hydrogels composed only of gelatin was maintained at baseline levels during the 3 weeks of the study, the cells in the GEL_HA systems gradually increased the secreted ALP. The activity determined at the end point for GEL_HA was significantly higher than that corresponding to GEL hydrogel and the value obtained in the first week of the experiment ($p < 0.0001$). Additionally, the expression of OPN was evaluated after 6 and 21 days of culture by immunostaining. The representative fluorescence images and the

correspondent quantitative analysis are shown in the Figure 6E and D. As expected, enhanced expression of this osteogenic marker was accomplished by mineralized hydrogels. OPN is a calcium-binding protein involved in the process of ECM calcification. The high levels of this marker are associated with the ECM mineralization process during the late osteogenic differentiation of osteoprogenitor cells ⁴⁸. These results confirm the osteoinductive properties of this mineralized biomaterial, presenting the capability to promote osteogenic differentiation of encapsulated hASCs without osteogenic supplements.

3.5 Morphological evaluation and biological performance of 3D biphasic systems

The native enthesis is characterized by progressive transition of the collagen fibers organization and mineral composition ⁴⁹. Collagen molecules alignment increases gradually towards the tendon tissue, whereas the mineral content increases towards the bone tissue. Therefore, in this work, the integration of gelatin-based mineralized hydrogels with anisotropic hydrogels results in the replication of the graded structural features related to the tendon-to-bone native interface.

Biphasic hydrogels developed by enzymatic assembling of the mineralized and anisotropic properties were assessed by μ -CT analysis to evaluate the integration between phases. As shown in the 3D reconstruction image (Figure 7A), a single continuous structure with well-integrated and smooth interface transition was successfully obtained. Interestingly, unlike other methods for obtaining biphasic scaffolds proposed for the regeneration of interfacial tissues, the remarkably simple and reproducible manufacturing method herein proposed, based on direct modelling

without the need of inter-phase coating process, resulted in the effective integration between the interfaces ¹⁹.

Figure 6. Physical and biological characterization of mineralized hydrogels. (A) Representative SEM images of freeze-dried gelatin hydrogels and hydroxyapatite (HA) containing systems. The arrows point the HA particles distributed around the structure (Scale bars: low magnification = 10 μm ; high magnification = 4 μm). (B) Young Modulus of the hydrogels obtained under

compression tests. Statistical differences: $p < 0.0001$. (C) Determination of alkaline phosphatase (ALP) activity secreted by hASCs after 1, 2 and 3 weeks of culture. Statistically significant differences between groups at each culture time are shown as * $p < 0.05$ and **** $p < 0.0001$. (D-E) Confocal images and quantification of the Osteopontin (OPN) expression (green) at 6 and 21 days of culture with nuclei and cytoskeleton stained in blue and red, respectively. Statistical differences: **** $p < 0.0001$ (scale bars = $75 \mu\text{m}$).

To evaluate the potential of the biphasic system to recreate the dissimilar physical and biological functions of the tendon-to-bone interface, a single hydrogel construct with hASCs embedded into both sides of the integrated phases was prepared and cultured for 21 days. A transition in the morphological cellular arrangement could be appreciated by nuclei and cytoskeleton staining and the subsequent analysis using confocal microscopy (Figure 7B). Whereas randomly distributed cells without preferential orientation were predominant in the mineralized phase, a clear cell alignment corresponding to the anisotropic section was displayed, as observed in monophasic hydrogels. These qualitative characteristics were confirmed by analyzing the directionality of the actin filaments (Figure 7D). Thus, a main sharp peak was obtained for the anisotropic phase distribution but multimodal flat distribution was achieved in the case of cells embedded in the mineralized phase. These differences observed in cell morphology are also reflected in their respective nuclei aspect ratio, which is higher for anisotropic section cells. (Figure 7C).

On the other hand, specific markers previously assessed using independent hydrogels units were also evaluated to further confirm their potential to induce distinct cell differentiation trends guided through the defined chemical and structural composition (Figure 7E-H). As expected, the

biphasic constructs showed a gradient of OPN and TNC deposition. Whereas higher expression of the osteogenic marker OPN was observed in the section corresponding to the hydrogel reinforced with HA, the TNC deposition was more prominent in the anisotropic phase. These results demonstrate that the functionalities of the gelatin hydrogels tuned by adapting their composition and manufacturing conditions were maintained in the integrated biphasic construct.

Figure 7. Evaluation of the biphasic hydrogels composed of mineralized and anisotropic phases.

(A) 3D reconstruction from micro-computed tomography images of the biphasic hydrogel presented on a coordinates axis (scale bar = 1 mm). (B) Representative confocal images of

biphasic hydrogel's interface with hASCs nuclei in blue and F-actin filaments in red after 21 days of culture. (C) Nuclei aspect ratio of cells in mineralized and anisotropic phases. (D) Directionality frequency plots of cytoskeleton alignment analysis. Osteogenic differentiation-related marker osteopontin (OPN) (E and G) and tendon tissue-related marker tenascin (TNC) expression evaluation in each of the different phase (F and H) (scale bars = 100 μ m). Statistical differences: * $p < 0.05$ and **** $p < 0.0001$.

Actually, the potential to encapsulate cells within the biphasic hydrogel network is one of the main advantages offered by the proposed strategy. It is worth mentioning that due to the not cell-friendly conditions required for the production of anisotropic hydrogels by directional freezing technique, the incorporation of cells in such systems is limited to 2D seeding on the surface of the performed structures¹⁶. Consequently, the size and distribution of the pores of the system restrict the cell infiltration and ECM protein deposition. This dependence has been recently observed with hASCs seeded on biphasic silk fibroin scaffolds showing a porosity anisotropy gradient prepared by the freeze-casting method⁵⁰. Moreover, despite many attempts have been made for the development of monophasic anisotropic hydrogels produced through ice-templating technique^{51, 52, 52}, few designs have managed to effectively integrate dissimilar features gradients into a single unit^{50, 50, 53}. Additionally, the potential for *in vivo* injectability and the possibility of the *in situ* exposure to a magnetic field may help to promote the clinical translation of enzymatically crosslinked hydrogels for interfacial tissue engineering purposes. Although the efforts made in this work were focused on mimicking the tendon-to-bone enthesis structure, the versatility of the gelatin-transglutaminase enzyme tandem for the development of multiple interfaces, such as cartilage-to-bone interfaces, can be envisioned.

In general, engineered constructs aimed at tissue engineering must incorporate in their design requirements the use of non-complex and reproducible preparation methods, be easy to sterilize and cost effective, among others to move forward to clinical translation ⁵⁴. Herein, a simple and reproducible processing method to fabricate a single scaffold with well-integrated phases is reported. It is especially important to bear in mind that gelatin, the main material used for the preparation of the structures, is already approved for human use ¹⁹. Furthermore, unlike many anisotropic hydrogels prepared by the alignment of magnetic nanomaterials under exposure of magnetic fields of the same order of those used in this work (hundreds of mT), the alignment of CNC in the hydrogel network without requiring magnetic iron oxide nanoparticles simplifies the synthesis and could improve the safety profile of the biomaterial ^{39, 39, 55, 56, 56}.

To further increase the potential of the proposed system to boost the healing process of the tendon-to-bone interface, the nanocomposite hydrogel could be functionalized with growth factors in order to stimulate the stem cells differentiation towards the specific phenotype ². Despite the optimal combination of these biochemical factors is not fully understood, the gradual combination of bone morphogenetic protein 2 with fibroblast growth factor 2 ⁵⁷ or transforming growth factor β 2 with growth/differentiation factor 5 ⁵⁸ have led to promising results. Although the evaluation of the incorporation of specific growth factors was beyond the scope of this work, the transglutaminase enzyme proposed in this research work presents the potential to support the covalent immobilization and the required sustained release of growth factors ^{59, 60, 60}. Additionally, the use of multi-chamber bioreactor devices could be an interesting approach to provide each region with customized and graded culture medium. Thus, the specific cellular environment may guide the differentiation of hASCs for *in vitro* applications.

4. Conclusions

In this study, we successfully developed an integrative 3D system, in which chemically and morphologically different sections have been assembled in order to generate gradients of mineralization and cellular alignment. Enzymatically crosslinked gelatin hydrogel served as the base material, incorporating fillers such as HA and CNC to provide advanced functionalities. While the inclusion of CNC and exposure of uniform magnetic field (400 mT) resulted in anisotropic structures that allow cell alignment and ECM protein deposition related to tendon tissue, the presence of HA particles generated stiffer hydrogels with feasibility to induce the differentiation of hASCs to osteogenic lineages. The design and versatility of these systems could be derived in both injectable formulations and implantable 3D structures. Overall, this methodology could be potentially useful for the development of unique 3D systems with mineralization and/or cellular organization gradients that are specially interesting for engineering interfaces such as connections between fibrous, cartilaginous and calcified connective tissues of the musculoskeletal system.

ASSOCIATED CONTENT

Supporting Information

Triphasic hydrogel; polarized optical microscopy images; hASCs live/dead staining (PDF)

Biphasic hydrogel (AVI)

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