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Regenerative and resorbable PLA/HA hybrid construct for tendon/ligament tissue engineering.

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Abbreviated tittle for running head: PLA/HA construct for tendon/ligament engineering.

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Abstract

Tendon and ligament shows extremely limited endogenous regenerative capacity. Current treatments are based on the replacement and or augmentation of the injured tissue but the repaired tissue rarely achieve functionality equal to that of the preinjured tissue. To address this challenge, tissue engineering has emerged as a promising strategy. This study develops a regenerative and resorbable hybrid construct for tendon and ligament engineering. The construct is made up by a hollow poly-lactic acid braid with embedded microspheres carrying cells and an anti-adherent coating, with all the parts being made of biodegradable materials. This assembly intends to regenerate the tissue starting from the interior of the construct towards outside while it degrades. Fibroblasts cultured on poly lactic acid and hyaluronic acid microspheres for 6 hours were injected into the hollow braid and the construct was cultured for 14 days. The cells thus transported into the lumen of the construct were able to migrate and adhere to the braid fibers naturally, leading to a homogeneous proliferation inside the braid. Moreover, no cells were found on the outer surface of the coating. Altogether, this study demonstrated that PLA/HA hybrid construct could be a promising material for tendon and ligament repair.

Keywords: regeneration, braid scaffolds, microspheres, fibroblasts, coating

1. Introduction

The main task of tendons and ligaments is transferring forces between muscles and bones (tendons) or between bones and bones (ligaments), stabilizing the joints. Both tissues are made from highly specialized connective tissue, having physiological limits on the force they can support; when these limits are exceeded, the tissue can be damaged, resulting in pain and dysfunction of the organ [19]. The injuries of tendons and ligaments can come from accidents, sport activities, tumours or degenerative pathologies, affecting the quality of life of patients and having an important economic cost [8].

When the conservative treatment fails or a breaking occurs, the surgery is the most accepted solution to recover the functionality of the damaged tissue. Autografts and allografts lead to good results, but the morbidity at the donor site when the autografts are used, or the possibility of disease transmission when allografts are used remain still as unsolved problems. Synthetic prosthesis can be implanted in large defects, but the long-term behaviour of this prosthesis is not as good as it could be expected [36].

Actually, the tissue engineering is a promising emerging field focusing on the regeneration of the tissue. Recovering the functionality is not the sole objective, but regeneration of damaged tissue. Tissue engineering is looking for cells and new biocompatible materials with capabilities to regenerate the biological tissues, and different strategies have been used to achieve this purpose: gene and cell based therapies [1, 18, 22, 25, 35], delivery of growth factors [6, 13, 17, 23, 37], implantable fibers systems [9, 10, 11, 14, 21, 22, 24, 32, 34] and allograft decellularized tissue [7, 30].

Gene based therapies supply genetic material to alter the DNA and regulate the secretion of proteins in a natural way, for long time, and without excessive therapeutic dosages or the activation of an immune response.

Cell therapies have opened new possibilities for the treatment of tendon and ligament injuries. MSCs and human adipose stem cells (hASCs) implanted in vivo have shown the ability to integrate into the tissue damage and induce the synthesis of tissue-specific extracellular matrix improving tissue regeneration.

Growth factors have a strong influence on the healing process of tendon and ligaments. They can be directly supplied or as a consequence of the gene-based or cell-based therapies.

Related to synthetic scaffolds, poly-lactide (PLA) and poly-glycolide (PGA) have been widely used as components of braided structures. Their biocompatibility, mechanical properties and hydrolytic degradation make them good candidates for tendon and ligament prosthesis, both separately or copolymerized; their copolymers can be tailored to reproduce the features of native tissue. In vitro, a loss of mechanical properties has been observed along the early months of degradation, and then they could be used as components of biodegradable prosthesis if the degradation rate goes according the regeneration of the native tissue.

Previous studies of ours described this idea[2, 3], consisting in a hybrid construct made from a synthetic and a biological component, intended for initially acting as a standard prosthesis, but able to regenerate the native tissue [3] and to be bio-resorbed in due time once the tissue is regenerated. We try to combine into this concept different features: immediate functionality (mechanical, geometrical) once implanted; progressive induced regeneration and organization of the tissue (neo-tissue formation) through cell supply; mechanical stimuli (growth factor release could also be considered); and final resorption of all the synthetic compounds.

This hybrid consists of a hollow braid of PLA acting as a load bearing element immediately after implantation. A set of poly-L-lactide (PLLA) and hyaluronan (HA) microspheres as

carriers of the transplanted cells is injected inside the braid. Microspheres as cell carriers have already been tested for another applications[12, 20]. The set of microspheres acts as a 3D scaffold within the braid lumen for the seeded cells. This scaffold on one hand ensures adhesion of the cells onto a surface, enhancing their proliferation and differentiation, and on another hand it is a flexible structure, capable of restructuring during the tissue growth process. Moreover, since the cells are seeded onto the microspheres, they are better protected against shearing and crushing during stretch than in case they were seeded on the braid fibers. In the literature other systems can be found as cell carriers, by encapsulating the cells in microspheres[16, 33, 38], but for our purposes, the cell carriers must play a biomechanical role, not always achieved if the cell carrier is an hydrogel or a degrading material. Moreover, since hyaluronic acid is a component of the ECM' ground substance, the HA microspheres may help to create an environment closer to the natural one while regeneration is taking place. On the exterior of the braid, a coating of HA must avoid adherences with the adjacent tissues, ensuring the gliding of the construct.

We already proved that the hollow braid of PLA can support the mechanical load when implanted, with similar behaviour to that of the natural tissue [2]. We have also proved that the degradation rate of the hollow braid could proceed according the regeneration of the tissue [4].

In this paper we establish the composition of the microspheres acting as cell carriers, and we test the performance of the assembled multicomponent construct in cell cultures, in order to assess cell viability and cell- materials interaction in a proof-of–concept situation.

2. Materials and methods

2.1. Materials

Poly-L-lactide (PLLA) Mw=124 kDa, Mw/Mn= 1.83, was purchased from Nature Works PLA, and Cargill Dow Polymers, Blair, NE, USA. Dichloromethane (CH_2Cl_2) CL0329 synthesis grade, was purchased from Scharlab S.L., Barcelona, Spain. Sodium hydroxide (NaOH) SO04181000, synthesis grade, was purchased from Scharlab S.L., Barcelona, Spain. From Sigma-Aldrich, St. Louis, MO, USA were purchased: Polyvinyl alcohol (PVA) (348406, 98% hydrolyzed, M_w 13000-23000 Da); Hyaluronan (HA), Hyaluronic acid sodium salt from streptococcus equi bacterial glycosaminoglycan polysaccharide (53747, Mw=1.63x106 Da); Divinyl sulfone (V3700 DVS, 97% purity); Isooctane (99% purity); Sodium bis(2-ethylhexyl) sulfosuccinate sodium salt, (323586, 98% purity); and 1-Heptanol (H2805, 98% purity).

2.2. Fabrication, characterization and cell culture of microspheres

2.2.1. Preparation of PLLA microspheres

PLLA microspheres were prepared by oil/water emulsion solvent evaporation technique. Briefly, PLLA was dissolved in dichloromethane (CH_2Cl_2) to obtain a 5 wt% transparent PLLA/CH_2Cl_2 solution. The solution was then added at 1 mL/min rate into deionized water containing 0.5% (w/v) polyvinyl alcohol (PVA) under agitation with a rate of 1350 revolutions per minute (rpm) by a mechanical stirrer. The agitation was continued for 24 h at room temperature to evaporate the organic solvent. The microspheres were collected after centrifugation at 4000 rpm for 5 min and then washed three times with distilled water to remove PVA. The recovered microspheres were freeze-dried and storage at -20°C until use.

2.2.2. Preparation of HA microspheres

HA microspheres were prepared by water in oil (W/O) emulsion and crosslinking method [26]. HA was dissolved in 0.2M of NaOH 0.2M to obtain a 5 wt% HA transparent solution. HA

solution was then added at 10 mL/h into the organic phase composed by isooctane and the surfactant, (1-heptanol 1.7 mL and sodium bis(2-ethylhexyl) sulfosuccinate). The emulsion was stirred at 1300 rpm during 15 min and then 112 µL of divinyl sulfone (DVS) were added in order to crosslink the HA microsphere. 20 minutes later the microspheres were collected by centrifugation and washed with acetone several times to remove the iso-octane. Finally, HA crosslinked microspheres were freeze-dried and storage at -20°C until use.

2.2.3. Particles size distribution

Particle size distribution of the microspheres was studied by laser diffraction particle size analyser Malvern Mastersizer 2000 (Malvern, United Kingdom) equipped with a Hydro 2000SM unit. Microspheres were suspended in ethanol (refractive index 1.36). Three measurements were carried out (n=3) and the size distribution was plotted as the average frequency percentage for each range of diameters. Average diameter and standard deviation were calculated for each type of microsphere.

2.2.4. Morphology

The surface morphology of PLLA and HA microspheres was observed by scanning electronic microscopy (JEOL JSM-5410, Japan). The microspheres were mounted on copper carriers with a graphite conductive strip and covered by sputtering with gold. Micrographs were taken at 10 kV.

2.2.5. Cell culture studies

Mouse fibroblasts from connective tissue (cell line L929) were used in the biological studies. PLLA, HA and mixtures of PLLA:HA microspheres in two different weight ratios, 2:1 and 1:2

were sterilized in ethanol 70% and conditioned in plain DMEM 4.5 g/l D-glucose (Fisher) overnight. After this time, 100 μL of L929 fibroblast cell suspension ($1x10^5$ cells/50mg microspheres) was mixed with the microspheres and incubated for 1 h at 37 °C and 5% CO_2 in order to ensure the early cell adhesion. Then, 900 μL of cell culture medium (Dulbecco's Modified Eagle Medium, DMEM (Fisher) 4.5 g/l D-glucose, supplemented with 10% of FBS (Fisher), 1% penicillin/streptomycin, 1% L-glutamine (Lonza)) was added to each tube. Each sample was cultured in triplicate during 1, 7 and 14 days (T1, T7 and T14). An Eppendorf tube with only cells was used as control. Culture medium was replaced every three days.

In addition, short-term cell culture studies (1, 3 and 6 hours) were assessed to determine the formation of cell-microsphere aggregates.

2.2.6. Cell viability

The viability of the fibroblast cultured on the microspheres and either in the construct during 1, 7 and 14 days was determined using the Cell Titer 96 Aqueous One Solution Cell Proliferation Assay MTS following manufacturer instructions (Promega). The samples were washed twice with PBS (Gibco) and incubated with plain DMEM (without phenol red) containing the MTS reagent (ratio 5:1) at 37°C for 3 hours in darkness. After the incubation time, the absorbance was measured by spectrophotometry at 490 nm with a microplate reader (Victor 3, PerkinElmer, USA).

2.2.7. Cell adhesion and morphology

Morphology and adhesion of L929 cells were analysed by scanning electron microscopy (SEM, Leica Cambridge S-360, UK). After each culture time, samples were washed several times with PBS and fixed in 2.5% glutaraldehyde (25% aqueous solution, Electron Microscopy

Science) for 1 h at 4ºC. After this time, the samples were washed several times with PBS and dehydrated in series of ethanol solutions (30%, 40%, 50%, 60%, 70%, 80%, 90%, 98% and 100%) for 15 min. Finally, the samples were dehydrated by the critical point technique and sputter-coated with gold for further analysis.

2.3. HA coating

The deposition of crosslinked HA onto the PLA braid was conducted by electrospinning using a single nozzle. Briefly, HA solution (0.28 g of HA solved in 7.98 g of distilled H_2O, 5.32 g of N,N-dimethyl-formamide and 1.48 g of absolute ethanol) and the crosslinker solution (DVS, 49.2 µL in 4.8 mL of NaOH 0.2 M), were loaded into 2 syringes, which were attached to two syringe pumps. A three-way union adapter with an extension tube was used to connect through Teflon tubing both pumps with a 26-gauge blunt end needle. The HA and crosslinker solution feeding rates were carefully set up at 5 and 1.6 $mL.h^{-1}$ respectively. 27 kV were applied between the needle and a metallic cylinder target, which was rotating around it axis at 120 rpm for 30 min.

2.3.1. Morphology of HA coating

The surface morphology and thickness of the dry HA coating was observed using a scanning electron microscope (JEOL JSM-5410, Japan). Micrographs were taken at 10 kV. The coated samples were positioned in the microscope longitudinally and transversely to examine the surface morphology and the thickness of the coating. Five samples were analysed (n=5) and the thickness was measured in three different areas of each of them. The thickness average was then calculated. In order to study the HA coating swelling behaviour in water, the

samples were swollen in water during 24h and then transversal images were analysed. The thickness of the coating was then calculated as described above.

2.4. Construct cell culture studies

The HA-coated PLA braid was sterilized in 70% ethanol during 5h. Ethanol was slowly changed to PBS with a dripping system in order to avoid the detaching of HA coating from the braid. Then, the HA-coated PLA braid was stabilized in PBS for 12 h and conditioned with DMEM for 24 h. Thereafter, the selected combination of microspheres (2PLLA:1HA) with previously cultured cells was injected inside the braid's lumen with a syringe. The constructs were then incubated for an hour at 37 ºC and 5% of CO_2. After this time, 10 mL of DMEM was added to the samples and cultured for 1, 7 and 14 days (T1, T7 and T14) at 37 ºC and 5% of CO_2. The culture medium was replaced every three days. Only cultured cells were used as a control.

2.5. Cell morphology

Cell distribution and morphology were examined by immunofluorescence analysis using a confocal microscope (Leica TCS SP2 AOBS, Germany). Samples were washed with phosphate buffer solution (PBS) for 5 minutes at room temperature, fixed in 3.7% of formaldehyde and incubated in the blocking-permeabilization buffer (8.9 mL of PBS (0.1 M), 1 mL of FBS (10%) and 0.1 mL of triton X-100 (0.1%)) for 2 h at room temperature. Then, the samples were staining with 1:50 bodipy-FL phalloidin (Invitrogen) and incubated overnight at 4ºC protected from light. Thereafter, the samples were washed three times with PBS every 5 minutes. Finally, the samples were mounted on microscope slides and a drop of DAPI-Vectashield (Vector Laboratories) was added in order to stain the nuclei.

2.6. Statistical analysis

Data were analysed using SPSS 16.0 software. Student's *t*-test and ANOVA were applied. The results are expressed as the mean ± SD. Significance was presented at *P*-values ≤ 0.05 as indicated in the figures.

3. Results

3.1. Construct assembly

PLA/HA construct developed in this study is made up by a PLA hollow braid with mechanical properties similar to native tendons [2]. In brief, the hollow braid was filling up with a mixture of PLLA and HA microspheres, as cell carrier, and crosslinking HA coating to avoid adherences from the surrounding tissue (Figure 1). L929 cells were cultured together with the microspheres and then injected into the hollow braid (Figure 1b). Figure 1d shows the microstructure of the HA coating. The average thickness of the coating layer was 33±5 µm in dry and 270 µm in swollen state respectively.

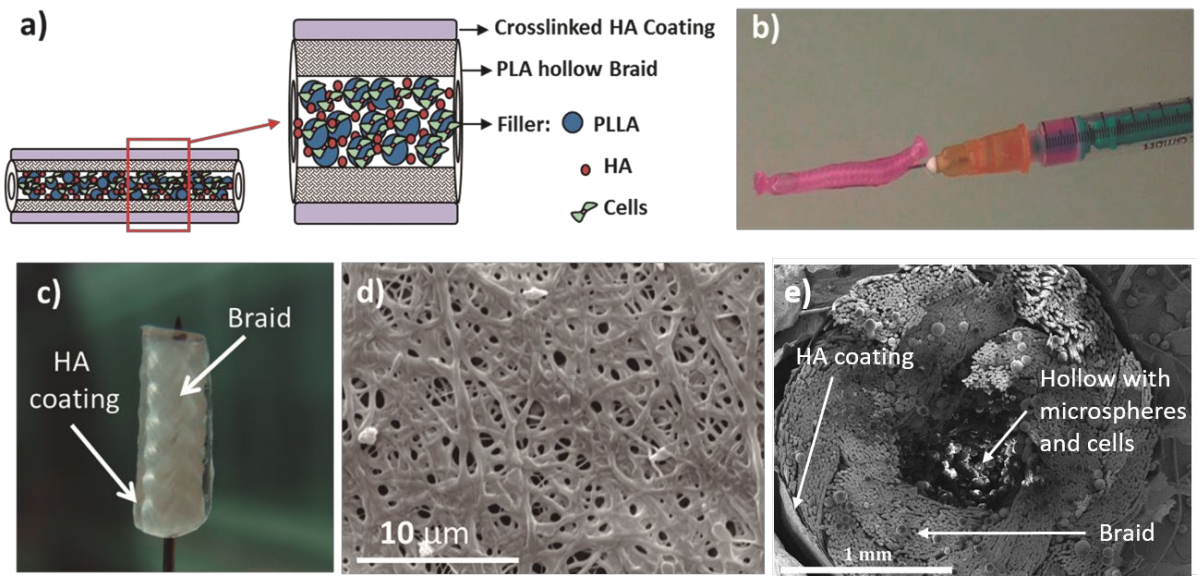

Fig. 1 Sketch of the construct. a) Overall view. b) Injection of microspheres and precursor cells inside the hollow braid. c) Macro-image (photograph) showing a segment of the construct. d) SEM image of the surface of the outer surface of the HA coating, showing its microporosity. e) SEM image of a cross section of the construct.

3.2. Microspheres production and cell culture

3.2.1. Size distribution and morphology of microspheres

Particle size distribution and morphology of the PLLA and HA microspheres were analysed. SEM micrographs showed a spherical shape and smooth surface of PLLA microspheres (Figure 2(a)), whereas HA microspheres exhibited an irregular shape and roughness surface due to dehydration during sample preparation (Figure 2(c)).

An average diameter of 110±80 μm was obtained for PLLA (Figure 2(b)) and of 20±14 μm for HA microspheres (Figure 2(d)). The histograms showed a broad size distribution, both in PLLA as in HA microspheres.

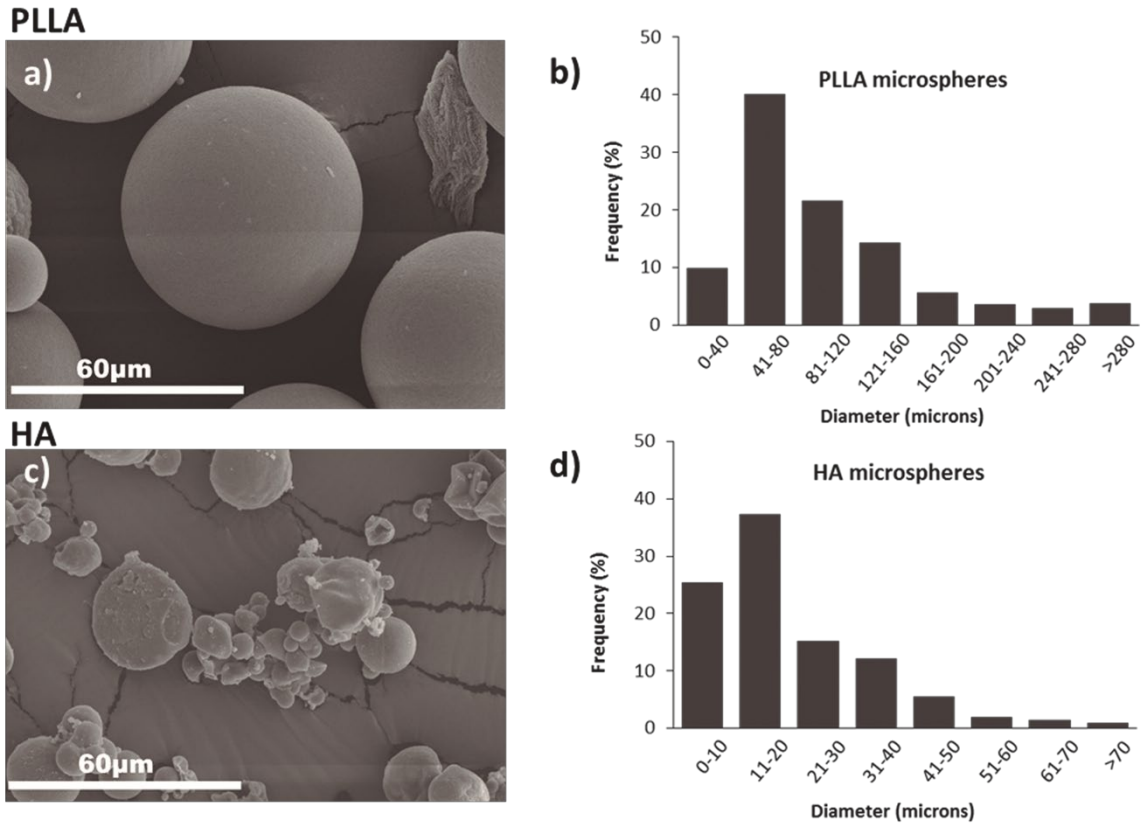

Fig. 2 Image of microspheres taken with SEM a) PLLA c) HA. Particle size distribution b) PLLA d) HA.

3.2.2. Cell viability, adhesion and morphology of L929 cells on both types of microspheres and on their mixtures.

Cell viability of L929 cells was determined through the colorimetric MTS assay (Figure 3). At one day of culture (T1), there are more viable cells on the PLLA microspheres than on the other tested groups, as expected, since this is the most cell-adhesive material. However, after 14 culture days the number of viable cells on the groups of microsphere mixtures (2:1 and 1:2 PLLA: HA rate in weight) was higher than on PLLA and on HA microspheres, and even higher than on the control group. Through an ANOVA analysis the cell viability of the two mixtures of microspheres for each culture time was compared: no significant differences

were found between T1 and T7, but significant differences were found after 14 days of culture for the 2 PLLA: 1 HA mixture.

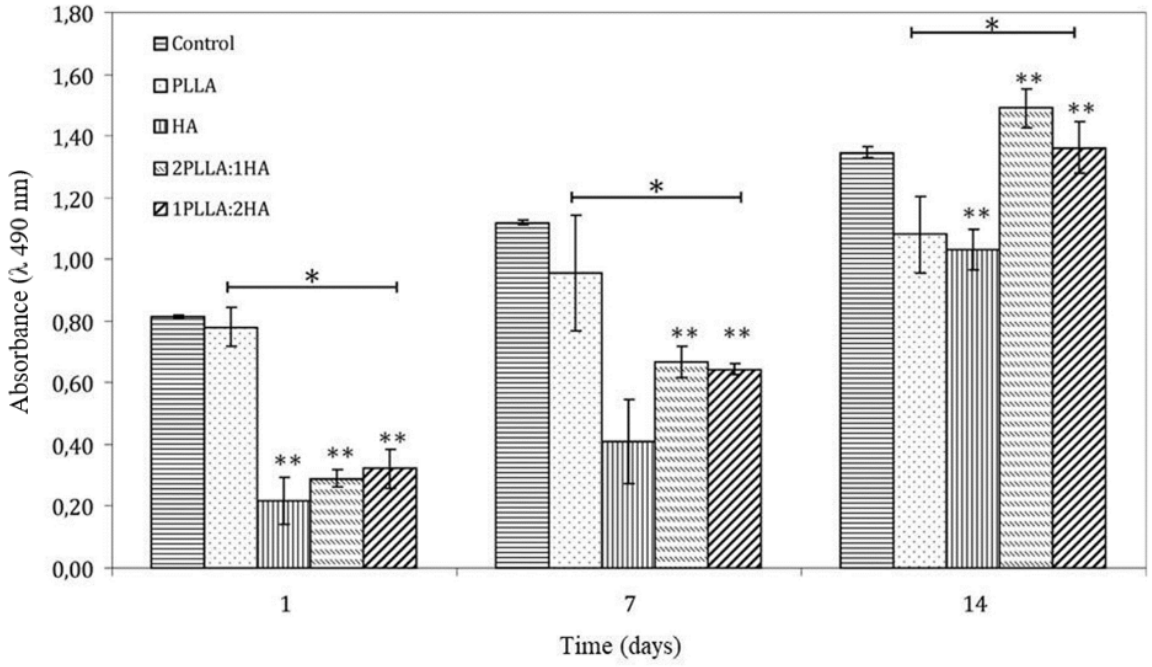

Fig. 3 Cell viability of L929 fibroblasts measured through colorimetric MTS assay on PLLA, HA microspheres and their mixtures (2:1 and 1:2) for T1, T7 and T14 (Values were means ± SD. (n=3) (* $p \leq 0.05$ groups vs. control at the same culture time) (** $p \leq 0.05$ for the same sample at different culture times).

At T1 the cells on PLLA microspheres have a more extended shape (spindle cells), with a high number of cells adhered on the surface. This observation is consistent with the results of the MTS assay. On HA microspheres at T1, a much lower number of cells are observed. At longer culture times the cells proliferate and adhere to the surface of PLLA, showing a well extended cytoplasm, whereas on HA microspheres the cells proliferate into cell-microsphere aggregates, with a rounded morphology.

The cells cultured on 2PLLA:1HA and 1PLLA:2HA mixtures of microspheres revealed similar morphologies to those on HA and PLLA microspheres, building up cell-microsphere

aggregates. In both systems, the rounded or extended morphology can easily be distinguished after 7 days of culture by SEM (Figure 4).

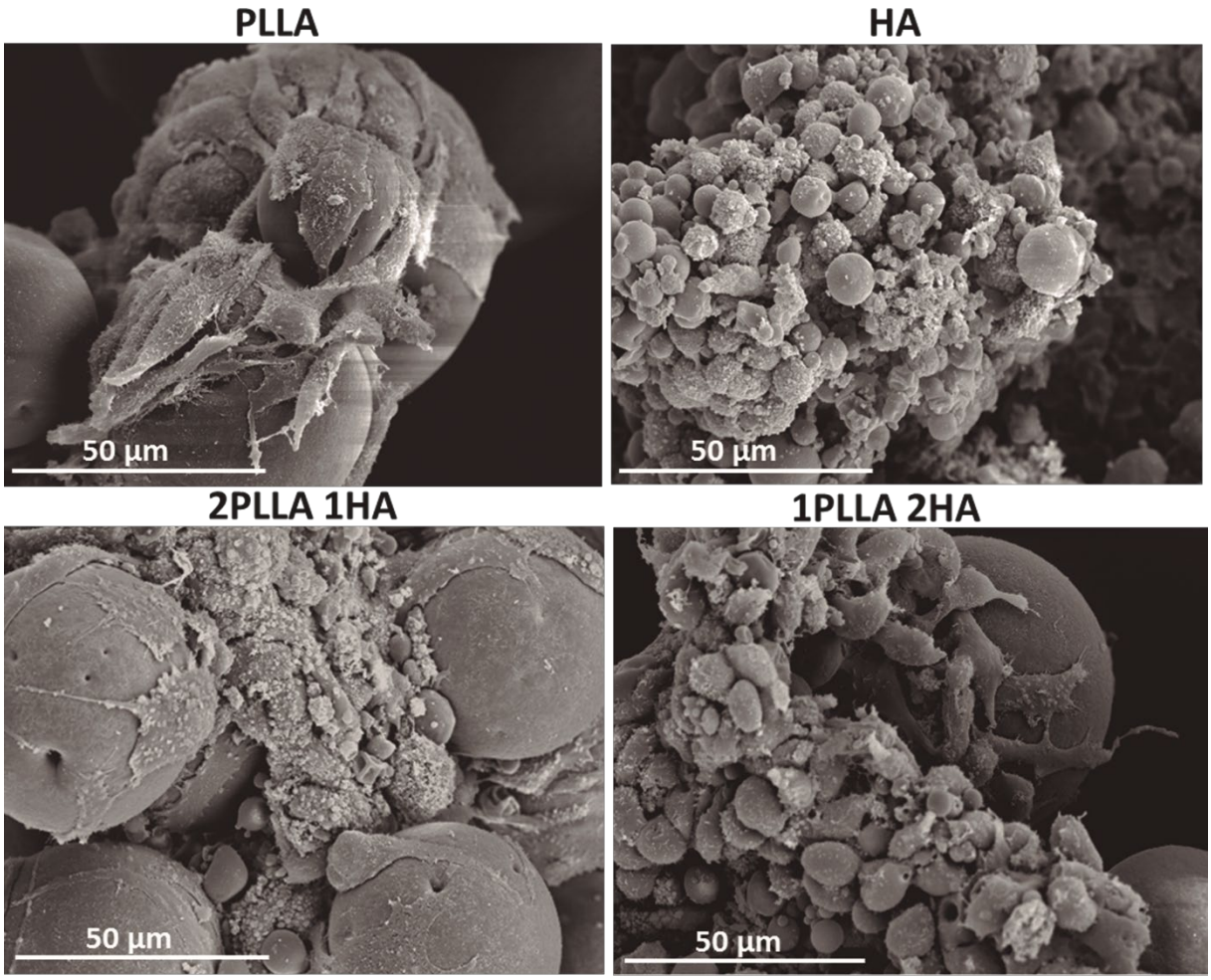

Fig. 4 SEM micrographs of L929 fibroblasts cultured on PLLA and on HA microspheres and their mixtures after 7 culture days.

Since the combination of 2PLLA:1HA showed the highest long term cell viability among all microsphere cultures, that mixture of microspheres was the one chosen to be injected into the PLA braid.

3.3. Early adhesion time of cells on microspheres.

In order to assess the adhesion time of cells on microspheres, SEM micrographs were obtained after 1, 3 and 6 h of culture. The evaluation of a time threshold is important in deciding how long the cells can be grown on the microspheres, to avoid the formation of aggregates and to prevent the subsequent obstruction of the needle during the injection. As can be seen on Figure 5, after 6 h, cellular microsphere aggregates start to appear indicating that times shorter than 6 h are convenient. Therefore, we set a culture time of 4 hours before the injection of microspheres and cells into the braid.

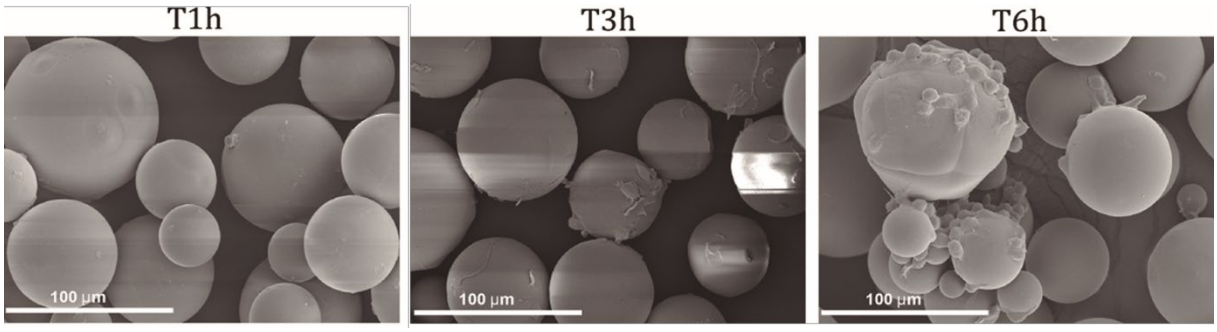

Fig. 5 SEM micrographs of PLLA microspheres cultured with L929 along 1, 3 and 6 hours.

3.4. Cell culture in the construct

The results of MTS assays after culture of the L929 fibroblasts within the construct for 1, 7 and 14 days showed that the number of viable cells increases until day 7, being constant after this time until the end of the experiment (Figure 6(a)). The control shows a similar behaviour. Figure 6(b) shows a CLSM image of the inner part of the construct at T7. The cells carried by the microspheres have migrated to the braided fibre. The longitudinal shape of fibres can be easily distinguished and the cells that adhered to them developed abundant actin filaments (green colour).

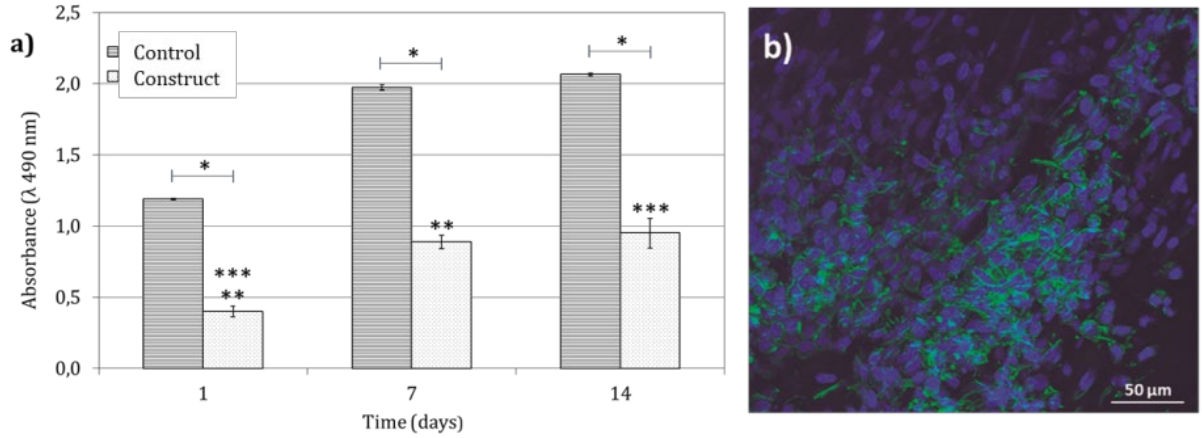

Fig. 6 L929 fibroblasts cultures on construct. a) MTS test (Values were means ± SD. (n=3). * $p \leq 0.05$ groups vs. control at the same culture time. ** and *** $p \leq 0.05$ for the same sample at different culture times. b) CLSM image of the interior of construct at T7. Cell nuclei are stained in blue (DAPI staining), and the F-actin fibres of the cytoskeleton in green.

Figure 7(a) shows a cross-sectional view of the construct after 7 days of culture. The internal section of the construct revealed several cells well attached both to the fibres and the microspheres, as well as abundant extracellular matrix production. The cells initially seeded on the microspheres migrated to the braided fibres (Figures 7(b)-(d)). The images also showed that the HA coating efficiently prevented cell leakage from within the construct's lumen, since no cells were observed on the HA coating (Figure 7(b)).

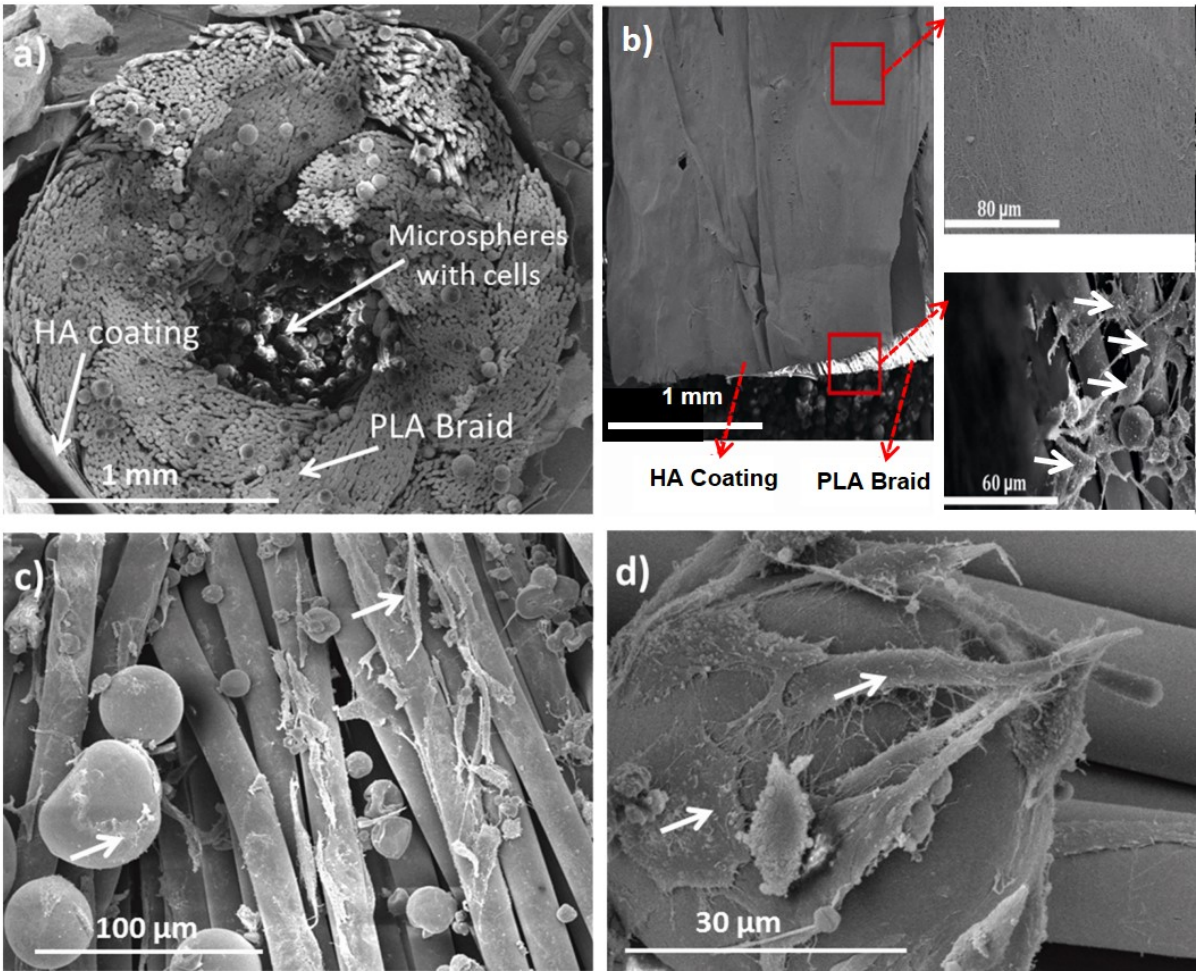

Fig. 7 SEM pictures of the interior of the construct after 7 days of L929 fibroblasts culture: a) cross section; b) Exterior and longitudinal view of the construct after a cross section, showing the cells adhered on the fibres and not adhered on the HA exterior coating; c) detail of PLA fibres with adhered cells; d) detail of cells adhered to microspheres. On (b), (c) and d) the white arrows indicate the cells.

4. Discussion

We have previously reported that the PLA braid used as the main load-bearing element in the design of the construct has a stress-strain curve that reproduces the nonlinear stress-strain behavior of natural tendons, with similar Young's modulus and toe region [2]. In addition, we have reported that the in vitro degradation process could be slow enough for

the regeneration of native tissue was produced before the construct loses its mechanical properties [4]. From the in vitro cell cultures performed it is clear that the PLLA microspheres are the ones that offer a better viability and adhesion for short time cultures, although for long time cultures, (14 days of culture), the presence of HA microspheres increased the viability and adhesion. Because of the hydrophobic PLLA surface, initially the cells adhere more easily to this material and their proliferation is slowed down [27]. The addition of the hydrophilic HA microspheres lead to a decreased cell adhesion, but a higher proliferation over time. HA is part of the extracellular matrix of the connective tissue, and its degradation products are angiogenic and promote injured tissue regeneration [5].

The cells adhered on the microspheres didn't produce large cell-microspheres aggregates during the first 4 h of culture. This is important in preventing occlusion of the syringe needle while injecting the cell-seeded carriers in the lumen of construct.

The morphology of cells attached on scaffolds depends on different factors such as the topography of the surface, or the hydrophobic/hydrophilic nature of the material [31]. Generally speaking, too hydrophilic surfaces are less cell-adherent because of the impossibility of extracellular matrix proteins to attach stably on them, while more hydrophobic surfaces are better cell adherents. Our 2PLLA:1HA microsphere combination as cellular carrier provided an efficient synergy of cell adhesion, survival and proliferation, showing after 14 days culture the microspheres' surfaces completely covered by cells and extracellular matrix. The cells thus transported into the lumen of construct were transferred to the braid fibers naturally, leading to a homogeneous proliferation inside the braid. The cells on the fibers will thus be mechanically stimulated when the construct becomes stretched once implanted. We expect that meanwhile the native tissue is regenerated and the construct degrades, a transfer of the mechanical load occurs to the newly generated

tissue, thus producing and orienting the collagen fibres in the direction of the applied load [15]. This mechanical stimulation also produces the enhancement of cell-differentiation processes towards phenotypes of load-bearing tissue cell types [30] and also an alignment of the cells in the direction of the stretching [15]. In fact, it has been proved that the nonlinear mechanical behavior of a ligament's prosthesis enhances the phenotype of pig fibroblasts [29], but in vitro and in vivo tests with mechanical stimulation should be performed to further check this hypothesis.

The absence in our experiments of cells on the outer surface of the HA coating suggests that this hydrophilic layer could be effective in impeding cell migration from the interior of the lumen toward the exterior; because of the small size of the pore (lower than 10 μm, as can be seen on figure 1 d). But also the non-adherent nature of the HA could avoid the finding of cells on the surface of HA coating. This is an important property for the prevention of possible tissue adherences on the construct once implanted, a problem that surgeons are familiar with in cases of tendon surgeries. Anyway, the hydrogel nature and the microporosity of this HA coating allows for free diffusion of biological fluids and nutrients[28].

Further in vivo and in vitro experiments should be carried out in order to verify the hypothesis stated in this paper. Cell culture with hMSCs could assess the viability, proliferation and differentiation of seeded cells, and also the effects of the mechanical stimulation on the regenerated tissue.

Our results show that the multiple-component construct described in this paper is a good candidate for a resorbable and regenerative prosthesis for tendon and ligament. The braid of the prosthesis would allow, when transplanted, an immediate load-bearing function, while protecting the viability of the cell supply. The cell supply would be conveyed on a mixture of PLLA microspheres acting as cell carrier and HA microspheres acting as filler and improving

the tissue regeneration. It is expected that the load transfer from the braid to the transplanted cells will direct their differentiation towards the tissue-specific lineages. An exterior coating of HA is intended to prevent adherences of the surrounding tissues, permitting a smooth gliding motion of the prosthesis.

Limitations

Even though the construct described in this work could be the starting point of a prosthesis for tendon and ligament, several issues must be still addressed.

More adequate cells (HMSCs cells) should be cultured instead the mouse fibroblasts, and also dynamical tests with mechanical stimulation on a bioreactor should be performed. In this way, the differentiation towards tenocytes could be evaluated as a consequence of the stretching.

Until now we have only evaluated degradation in vitro, trying to reproduce the natural conditions, but in vivo degradation tests should be carried out to match the degradation rate of the construct to the regeneration rate of the natural tissue.

To finish, the surgery to attach both endings of the construct to bone and muscle is still an unsolved problem.

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The authors declare that they have fulfilled ethical standards.

The authors declare that they have no conflicts of interest.

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