Customised multiphasic nucleus/annulus scaffold for intervertebral disc repair/regeneration

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In the case of a degenerated intervertebral disc (IVD), even though spinal fusion has provided good short-term clinical results, an alteration of the spine stability has been demonstrated by long-term studies. In this context, different designs of IVD disc prostheses have been proposed as an alternative to spinal fusion. However, over the past few years, much of the recent research has been devoted to the IVD tissue engineering, even if several limitations related to the complex structure of IVD are still presented. Accordingly, the aim of the current paper is to develop a strategy in designing customized multiphasic nucleus/annulus scaffolds for IVD tissue engineering, benefiting from the great potential of reverse engineering, additive manufacturing and gels technology. The device consisted of a customized additive-manufactured poly(ε-caprolactone) scaffold with tailored architectural features as annulus and a cell-laden collagen-low molecular weight hyaluronic acid based material as nucleus with specific rheological and functional properties. Analyses on the developed devices demonstrated appropriate rheological and mechanical properties. Preliminary biological analyses demonstrated that human mesenchymal stem cells were viable over the culture period.

Key words: Polymers; Reverse Engineering; Additive Manufacturing; Gels; Intervertebral disc; Tissue Engineering.

Running Head: 3D scaffolds for IVD tissue engineering

Introduction

Spine represents a cooperative system of elements which has a unique function. It provides trunk flexibility and protects the spinal cord and nerve roots which pass through the spinal canal and foramen.

As frequently reported, spinal diseases represent a serious medical problem affecting many people in the world and almost all of the spinal components (e.g. vertebral bodies, ligaments, intervertebral discs, facets and laminae) can be dissected at surgery, depending upon the deformity or pathology[1,2]. Intervertebral discs (IVDs) provide flexibility to the spine enabling the body to twist and bend into a wide range of postures.

IVD is a complex tissue made of a “nucleus pulposus”, which is a gel-like matrix mainly consisting of water, collagen and proteoglycans, surrounded by the “annulus fibrosus”. The annulus presents a layered structure where each layer is reinforced by a regular pattern of collagen fibers[1,2]. Vascularization is negligible in the annulus and nucleus regions, whereas it is moderate at the vertebral body level.

IVD degeneration generally involves dehydration of the nucleus which is also accompanied by small tears in the annulus. This is a common reason for the back pain which affects many people in the world. The pain may become a chronic disabling condition[3,4].

In the case of a degenerated disc, medical practice today involves different devices (i.e, interbody spacer, disc prosthesis) to restore spine stability and function.

Different types of interbody spacers have been designed and produced, such as ring-like, rectangular, or tapered and hollow threaded cylindrical cages[5-7]. However, fusion sill presents some limitations such as biomechanical alteration of adjacent vertebrae, symptomatic hypertrophic facet arthroplasty, spinal stenosis, and osteophyte formation. Furthermore, many phenomena such as loss of spinal mobility, graft collapse resulting in suboptimal sagittal balance, autograft harvest site pain, and alteration of muscular synergy are correlated to fusion treatment. For this reason, over the past few years, a great attention has been focused on the restoration of flexibility through the replacement of a damaged IVD with a

prosthesis. Current prostheses are characterized by standard sizes and generally consist of a polymer core (i.e., ultra-high molecular weight polyethylene) interposed between two metallic plates. Anyway, many efforts have also been made in the design of a poly(2-hydroxyethyl methacrylate)/poly(methyl methacrylate) semi-interpenetrating polymer network (semi-IPN) composite hydrogel reinforced with poly(ethylene terephthalate) fibers as nucleus/annulus substitute, which was able to reproduce the mechanical and mass transport properties of the natural IVD, also optimizing its characteristics (i.e., shape, size, compressive modulus and strength) at several locations along the spine. Specifically, the biomimetic approach allowed to develop a nucleus/annulus synthetic system with a softer and more hydrophilic inner core and a stiffer and less hydrophilic outer fibrous structure[8,9].

A pilot-scale production of a biomimetic and customized total IVD prosthesis was also proposed by Gloria et al. (2011, 2012) integrating computer tomography, filament winding and moulding technologies[2,10].

In this field much of the recent research has been devoted to the “biologic restoration” of the IVD using different approaches (i.e., tissue engineering, growth factors, gene therapy)[11]. However, these approaches still present several limitations especially related to the complex structure of IVD, consisting of three tissues (nucleus, annulus and cartilage endplate) which differ physiologically, chemically and histologically. IVD is populated by vacuolated notochordal cells and/or chondrocyte-like cells in the nucleus pulposus, fibroblast-like cells in the annulus and chondrocytes in the endplates[12].

Tissue-engineered replacement for the nucleus or the whole IVD have been already considered using several technologies, biomaterials and cell sources[3,11-24]. The annulus and the nucleus tissues were engineered, and the anatomic shape of IVD was reproduced[25]. A totally natural IVD structure was developed by Bowles et al. (2011)[21]. In particular, such studies represented a breakthrough in comparison to the classical approaches which focused on purely mechanical devices designed to either eliminate or enable flexibility of the diseased motion segment. The feasibility of engineering a

functional spinal motion segment was demonstrated by the authors taking into account biological therapies for degenerative disc disease[21]. The tissue-engineered IVD consisted of a gelatinous nucleus pulposus surrounded by an aligned collagenous annulus fibrosus. When implanted into the rat caudal spine, tissue-engineered IVD maintained disc space height, produced *de novo* extracellular matrix, and integrated into the spine, yielding an intact motion segment with dynamic mechanical properties similar to that of native IVD[21].

Similarly, Moriguchi et al. (2017) designed a tissue-engineered IVD scaffold for canine cervical spine[22]. IVD components were constructed using adult canine annulus fibrosis and nucleus pulposus cells seeded into collagen and alginate hydrogels, respectively[22]. Seeded gels were formed into a single disc unit using 3D printed of UV-curable watertight acrylic plastic molds designed from the geometry of the canine spine. The results confirmed the ability of engineered intervertebral discs to re-establish many aspects of the native disc maintaining their position, structure and hydration as well as disc height over 16 weeks *in vivo*[22].

Martin et al. (2017) fabricated by electrospinning a nanofibrous scaffold to match the rat caudal disc geometry[23]. Layers of poly(ε-caprolactone) (PCL) nanofibers and poly(ethylene oxide) were sequentially electrospun onto a rotating mandrel as aligned multilayer sheets. PCL was selected for the this region because of its slow degradation rate, robust mechanical properties, and its ability to be fabricated via electrospinning into ordered structures that replicate the fiber architecture of the native tissue. The scaffold was cut at an angle into strips in which fibers ran 30° to the strip length, replicating the structure of an individual lamella in the native annulus fibrosus. Strips with alternating alignment were wrapped around a post into concentric discs with final dimensions 4–5 mm diameter and 2 mm height. Scaffolds were coated with a 20 μg/mL fibronectin solution to improve cell attachment. The nucleus polposus portions of the scaffolds were generated using a photocrosslinkable bioactive hydrogel: methacrylated hyaluronic acid, to recapitulate the highly hydrated state of the native tissue. Results in short-term studies showed that the scaffolds were able to preserve their

structure, prevent intervertebral bony fusion, and match native disc mechanical function at physiologic loads *in situ*[23].

The two different regions were successively combined with two acellular, porous PCL foams as endplate analogues. The long-term *in vivo* integration and mechanical function of the scaffolds in the rat caudal spine were demonstrated[24]. These engineered discs maintained composition and structure while functionally maturing *in vivo*, reaching near-native tensile and compressive mechanical properties by 20 weeks[24].

Interesting results were also obtained using meshes of a synthetic polymer such a bioresorbable polyester (polyglycolic acid coated with polylactic acid) combined with hydrogel (sodium alginate mixed with calcium sulphate) as annulus scaffold seeded with cells from sheep annulus[25]. The great potential of autogenic and allogenic mesenchymal stem cells was demonstrated in the case of IVD degeneration promoting partial regeneration in several animals models[12].

Recently, acellular and cell-laden methacrylated gellan gum hydrogels were analyzed for potential nucleus regeneration[26]. In this context, collagen-low molecular weight hyaluronic acid (LMW HA) semi-IPNs loaded with gelatin microspheres were also developed for cell and growth factor delivery, providing promising results[27].

Chitosan is one of the natural polymers which has attracted much recent attention since it exhibits excellent biological properties such as biodegradability, antibacterial, immunological and wound-healing activities. It has also been considered an interesting candidate as a support material for cell culture, gene delivery, and tissue engineering. As an example, bone marrow-derived mesenchymal stem cells (BMSCs)-chitosan hydrogel complex was studied as tissue-engineered nucleus pulposus providing an experimental approach for the clinical application[28].

A further design involved the use of cellulose nanofiber-reinforced chitosan hydrogel composites for intervertebral disc tissue repair, especially in the case of annulus tissue defects when used as patches against disc nucleus protrusion, however functioning as support for IVD regeneration[29].

On the other hand, the introduction of additive manufacturing processes (i.e., 3D fiber deposition) allowed for the fabrication of customised scaffolds for tissue engineering from computer aided generated models, thus offering the possibility to control the design, porosity, interconnectivity of the structures, and, hence, the mass transport properties (i.e., diffusivity, permeability)[30].

In this context, a methodology for producing patient-specific scaffolds which reproduce the annulus fibrosus of the human IVD by means of the combination of magnetic resonance image (MRI) and 3D bioprinting[31] as well as custom-tailored tissue-engineered PCL scaffolds for total disc replacement[32] were also proposed. Accordingly, a first design of customized devices for potential IVD tissue engineering, consisting of 3D printed PCL scaffolds and collagen-LMW HA-poly(ethylene glycol) ether tetrasuccinimidyl glutarate (4S-StarPEG) materials, loaded with chitosan nanoparticles (CNPs), was reported in the present paper.

Materials and Methods

Synthesis of Chitosan nanoparticles (CNPs)

Chitosan 1 % (w/v) in 1 % (v/v) acetic acid was filtered using a pore membrane (0.8 μm, Millipore) and diluted (1:4) with methanol. Succinic anhydride (≥ 99 % GC) was dissolved in 5 mL of acetone at 4 % (w/v) and dropwise added under magnetic stirring at 20 °C. The solution was left overnight[33]. The formed gel was removed, properly diluted in methanol and dialysed against ultrapure water for 3 days. The precipitate was collected by centrifugation and lyophilised (Suc-chi). Suc-chi (aq) (1 % w/v) was mixed with liquid paraffin (x10eq), homogenised for 30 minutes at 24000 rpm. Successively, Span 85 solution was added (0.1 volume eq.) and homogenised. 1 M NaOH solution (0.3 volume eq.) was dropwise added and the solution was homogenised, properly diluted in toluene and washed by centrifugation (3000 rpm). The above reported procedure was repeated using ethanol and collected in water and lyophilised, obtaining CNPs[33]. Chitosan, succinic anhydride and all the reagents to prepare CNPs were purchased from Sigma Aldrich.

Preparation of collagen-LMW HA-4S-StarPEG-CNPs and cell-laden hydrogel

Collagen-LMW HA crosslinked by 4S-StarPEG (Sigma Aldrich)[34] loaded with CNPs was prepared according to a protocol similar to that already described in a previous work for the preparation of composite collagen-LMW HA semi-IPNs[27]. In brief, sterile LMW HA powder (M_w = 150–300 kDa, Anika Therapeutics, Abano Terme, Italy) dissolved in Dulbecco′s Modified Eagle′s Medium (DMEM, Sigma Aldrich) was added to sterile collagen solution (Vitrogen) in order to provide a final LMW HA concentration of 2.5 mg/mL and collagen concentration of 1.2 mg/mL. CNPs were added to the mixture to the final concentration of 0.025 mg/mL. 4S-StarPEG (1 mM) was also added and the solution was then incubated at 37°C for 1 h.

With regard to the preparation of cell-laden collagen-LMW HA-4S-StarPEG-CNPs, human mesenchymal stem cells (hMSCs, Lonza, MD) were directly incorporated into the mixture. hMSCs; ($1x10^6$ cells/mL), used at passages 4–6 from primary culture, were cultured in α-MEM (Bio-Whittaker, Belgium) containing 10 % (v/v) FBS (GibcoTM, Thermo Fisher Scientific), 100 U/mL penicillin and 0.1 mg/mL streptomycin (HyClone, UK), in a humidified atmosphere at 37 °C and 5% (v/v) CO_2.

All steps were carried out under sterile conditions.

Cell-laden multiphasic nucleus/annulus scaffolds

A micro-computed tomography (micro-CT) was performed through a SkyScan 1072 system (Aartselaar, Belgium), at a resolution of 5.8 μm, to capture the image and, hence, shape, and size of porcine IVDs (female Large White/Landrace cross pigs, 11.1±1.0 kg), using a rotational step of 0.9° over an angle of 180°.

Cross-sections and 3D model of IVD were reconstructed using Skyscan's software package (Figure 1a). The obtained images were first smoothed, processed for ring artefact reduction and beam hardening correction, and then saved as 2D stack images.

The images were properly aligned and 3D modelling process was carried out. The acquired image

dataset was used to generate the 3D model, covering the pixels which enclosed the areas of interest in each 2D image. The 3D model was smoothed in order to reach an appropriate resolution and the STL file was exported.

Starting from STL file, 3D scaffolds were fabricated by 3D fiber deposition technique, using a 3D plotter (Bioplotter dispensing machine, Envisiontec GmbH, Germany) equipped with a Computer-Aided Design (CAD)/Computer-Aided Manufacturing (CAM) system.

PCL pellets (Aldrich, M_w=65000) were initially placed in a stainless steel syringe and, then, heated at a temperature of 120 °C through a cartridge unit placed on the mobile arm of the 3D plotter. As PCL reached the molten phase, a nitrogen pressure of 8-8.5 bar was applied to the syringe through a cap. 3D scaffolds were manufactured by alternatively extruding and depositing the PCL fibers using a specific lay-down pattern (0°/0°/90°/90°). Many layers were suitably designed and manufactured to obtain a structure for the annulus region.

The scaffolds were characterized by the fiber diameter (depending on the needle diameter – 500 µm and/or the deposition speed – 50 mm/min), fiber spacing (strand distance, i.e. center-to-center distance – 750 µm) and layer thickness (400 µm), which influence the overall pore size, the mass transport properties and the mechanical performances.

Images of customized 3D PCL scaffolds for annulus tissue engineering are reported in Figure 1b.

Figure 1. a) Results from micro-CT performed on porcine IVD, b) Images of 3D additive-manufactured PCL scaffolds for annulus tissue engineering. Frontal view (left) and top view (right). Scale Bars: 10 mm.

A further model of a total custom-made IVD was also developed including end-plates, even if they did not fully reproduce the structures of the naturals ones (Figure 2).

Figure 2. Different images of the custom-made IVD scaffold, showing the (a) non-porous outer layer, b) the porous superior endplate, c) the non-porous inferior endplate. Scale Bars: 10 mm.

The superior porous endplate was obtained by using a temporary water-soluble support that was placed into the empty nucleus region in order to allow the fiber deposition during the manufacturing process. The temporary support was then removed.

Cell-laden collagen-LMW HA-4S-StarPEG-CNPs was used to properly fill the nucleus and annulus regions of the 3D additive-manufactured PCL scaffold, by promoting gel formation into the structure, whose preparation was previously described. To this aim, 3D printed scaffolds were sterilized by soaking first in 70 % (v/v) ethanol (1 h) and then in 1 % (v/v) antibiotic/antimycotic in phosphate-buffered saline (PBS, Sigma Aldrich) (2 h), and prewetted in medium (2 h).

Characterization of collagen-LMW HA-4S-StarPEG-CNPs hydrogel

The viscoelastic properties of collagen-LMW HA-4S-StarPEG with and without CNPs were evaluated at 37 °C using a rheometer (Gemini, Bohlin Instruments) with parallel-plate geometry. In particular, serrated parallel plates were employed to avoid slippage.

The linear viscoelastic region was determined and small amplitude oscillatory shear tests were carried out. The frequency was varied from 0.01 to 2 Hz. The storage modulus or elastic modulus (G') and loss modulus or viscous modulus (G'') were properly evaluated in the analyzed frequency range.

Steady shear measurements were also performed to evaluate the viscosity as a function of the shear rate at a temperature of 37 °C in a wide range of shear rate (0.01–100 s^{-1}).

Injectability tests were carried out on collagen-LMW HA-4S-StarPEG-CNPs at 37 °C using a syringe with a 4.5 mm inner diameter and a 16 G needle. Briefly, a syringe was loaded with the material and suitably mounted on an INSTRON 5566 testing machine. According to a procedure already used for injectable semi-IPNs in previous studies[27], a piston speed of 40 mm/min was employed and the applied load for injecting the materials into and through the 16 G needle was measured. An empty syringe was also analyzed as control group, also providing information on the friction between the piston and the syringe walls[27].

Characterization of cell-laden multiphasic nucleus/annulus scaffolds

Compression tests were carried out on cell-laden customized multiphasic nucleus/annulus scaffolds and natural IVDs at 1 mm/min using an INSTRON 5566 testing machine.

Porcine natural disc height, lateral width and anterior-posterior width (8.2 ± 1.2 mm, 27.1 ± 3.0 mm and 17.6 ± 2.1 mm, respectively) were measured by caliper, optical photogrammetry and also confirmed by micro-CT analysis.

The segments were properly isolated from each spine and prepared by properly removing posterior elements as well as ligamentous and muscular structures, following a procedure already reported in the literature[10]. A horizontal cut was made through the middle of the two adjacent vertebrae. Thus, IVDs with upper and lower semi-vertebral bodies were placed between two parallel stainless steel plates and compressed.

All the tests were performed in a watery environment and at 37°C.

Compressive modulus was evaluated from the slope of the linear region of the stress-strain curves.

Cell-laden multiphasic nucleus/annulus scaffolds for IVD tissue engineering were also preliminarily analyzed after crystal violet staining (Sigma Aldrich). Briefly, hMSCs were stained with crystal violet dye, which binds to proteins and DNA and the 3D structures were analyzed using a stereomicroscope (Olimpus SZX7). The cell-laden scaffolds were fixed with 4 % (v/v) paraformaldehyde, rinsed twice with PBS buffer and incubated with crystal violet 0.1 % (w/v) at room temperature for 1 h. Cells that underwent cell death, lost their adherence and were subsequently lost from the population of cells, reducing the amount of crystal violet staining. After that time, the scaffolds were washed three times in PBS and observed by microscope.

Cell viability and proliferation were evaluated by using the Alamar Blue assay (AbD Serotec Ltd, UK). In particular, at 1, 3 and 7 days after seeding, the cell constructs were rinsed with PBS, and for each sample, 200 mL of DMEM without Phenol Red (HyClone, UK) containing 10 % (v/v) Alamar Blue was added, followed by incubation for 4 h at 37°C. A specific volumetric amount of solution was then removed from the wells and transferred to a new 96-well plate. The optical density was

measured with a spectrophotometer (Sunrise; Tecan, Männedorf, Zurich, Switzerland) at wavelengths of 570 and 595 nm. Each experiment was performed at least three times in triplicate.

Statistical analysis

Rheological, injectability and mechanical data were analyzed using t-Student test, whereas ANOVA followed by Tukey's test was used for biological analysis ($p<0.05$).

Results and Discussion

The aim of the present paper was to develop customized nucleus/annulus scaffolds for intervertebral disc tissue engineering, consisting of cell-laden collagen-LMW HA-4S-StarPEG-CNPs and PCL structures with tailored architectural features.

With regard to the nucleus regeneration, a previous paper proposed an intriguing strategy based on the TGF-β3 delivery by gelatin microspheres embedded in a collagen-LMW HA semi-IPN[27].

Furthermore, different multi-arm PEG-based hydrogels were also analyzed as injectable systems in the field of tissue engineering[35] and, among the crosslinking methods, the potential of 4S-StarPEG on the structural, physical and biological properties of collagen fibers was already demonstrated[36].

Thus, benefiting from the previous studies and our experiences in the field, a specific formulation of collagen-LMW HA-4S-StarPEG-CNPs was selected to design the cell-laden gels. The concentration of CNPs was optimized to achieve viscoelastic properties that should be still suitable for the specific application after the injection through a 16 G needle.

Results from small amplitude shear tests highlighted that the values of storage and loss modulus increased with frequency. In addition, G' values were always higher than G'' ones in the investigated frequency range (Figures 3). Specifically, with regard to collagen-LMW HA-4S-StarPEG-CNPs, G' values ranged from 14.2 ± 1.1 Pa to 39.6 ± 2.1 Pa as the frequency increased from 0.01 to 2 Hz.

The presence of CNPs improved both dynamic moduli and the above reported G' values were significantly higher than those obtained for collagen-LMW HA-4S-StarPEG (i.e., from 11.8 ± 1.0 Pa

to 31.1 ± 2.3 Pa) (i.e., p=0.0070 at 0.01 Hz; p=0.0003 at 2 Hz) (Figure 3a).

Anyway, a decrease of both dynamic moduli was observed for collagen-LMW HA-4S-StarPEG-CNPs after the injection through a 16 G clinical needle, without altering the rheological behavior as G' was always higher than G''. In particular, in the analyzed frequency range, the injection led to G' values varying from 11.8 ± 0.8 Pa to 19.7 ± 1.3 Pa, which were lower than those found before the injection (from 14.2 ± 1.1 Pa to 39.6 ± 2.1 Pa) (Figure 3b). The observed differences were statistically significant (i.e., p=0.0043 at 0.01 Hz; p=0.0001 at 2 Hz).

Figure 3. Results from small amplitude shear tests in terms of storage (G') and loss (G'') modulus as function of frequency: a) effect of CNPs on collagen-LMW HA-4S-StarPEG, (n=5); b) effect of injection through a 16 G needle, (n=5).

A shear thinning behavior was demonstrated by steady shear tests as the viscosity of collagen-LMW HA-4S-StarPEG-CNPs decreased from 130.7 ± 12.8 Pa·s to 0.017 ± 0.001 Pa·s with increasing shear rate, thus supporting the possibility to inject the material (Figure 4a). In addition, load-displacements curves from injectability tests evidenced an initial linear region, a maximum load, dropping load and plateau-like region. The material was completely injected at the end of the plateau-like region (Figure 4b). The values of maximum load and plateau load provided important information on the initial resistance to flow into the needle and on the continuous flow of material through the needle, respectively. The values of maximum load (3.10 ± 0.30 N) and plateau load (0.65 ± 0.08 N) were significantly higher than those found for the empty syringe (2.20 ± 0.20 N and 0.45 ± 0.04 N) (p=0.0005 and p=0.0011, respectively). Furthermore, the findings demonstrated the possibility to easily inject the material into and through the clinical needle. Anyway, it is worth remembering that the use of a wide range of clinical needles (14-29 G) is widely described in the literature and the potential effects of the different diameters on the disc degeneration were evaluated[27]. In the present research, the effect of the injection on the viscoelastic properties and the functional injectability of collagen-LMW HA-4S-StarPEG-CNPs were analyzed using a typical needle (i.e., 16 G), which falls in the range of those already studied for nucleus replacement or regeneration.

Figure 4. a) Results from steady shear tests on collagen-LMW HA-4S-StarPEG-CNPs: viscosity as function of shear rate, (n=5); b) Results from injectability tests: Typical load-displacement curves, (n=5). Empty syringe (solid line) and injected collagen-LMW HA-4S-StarPEG-CNPs (dotted line).

On the other hand, Figure 5 reports stress-strain curves from compression tests on the cell-laden multiphasic nucleus/annulus scaffolds and natural IVDs up to a stress level of 4.0 MPa.

Figure 5. Typical stress-strain curves obtained from cell-laden multiphasic nucleus/annulus scaffolds and natural IVDs, compressed at 1 mm/min up to a stress level of 4.0 MPa, (n=5).

However, even though the structure of the designed device differs from the natural one, the obtained results clearly showed that the initial J-shaped stress-strain curve displayed by IVDs was reproduced. The typical high flexibility of the soft biological tissues and a high compressive strength were achieved[37]. In the natural IVD the initial upward concavity (toe region) is related to the gel-like matrix of the nucleus and to the rearrangement of the fibres, which straighten their crimped waveform and reorient themselves in the transverse direction. The linear region of the stress-strain curve is due to the fibres straightening as the mechanical contribution of the fibres strongly increases during the loading process[37]. Differently from the natural IVDs, the toe and linear region of the curve may be mainly related to the column-like behaviour and densification zone of 3D porous structures. Specifically, a further difference results in the stress transfer mechanism, as in the case of the cell-laden multiphasic nucleus/annulus scaffolds, the overall mechanical behaviour may be ascribed to both annular PCL scaffold and gel flow in the interconnected pore network, even if the former would provide the major contribution since the beginning of the loading process.

These differences led to a lower flexibility (i.e., lower toe region and, hence, final strain) of the developed devices, in comparison to natural IVDs. In particular, values of strain at the end of the toe region (0.05 ± 0.01 mm/mm) and final strain (0.10 ± 0.01 mm/mm) for the cell-laden multiphasic nucleus/annulus scaffolds were significantly lower than those found for natural IVDs (0.08 ± 0.01

mm/mm and 0.13 ± 0.01 mm/mm) (p=0.0021 and p=0.0015, respectively). However, even though the developed systems displayed a compressive modulus of 86.3 ± 4.0 MPa, which was higher (p=0.0311) than that found for natural IVDs (75.1 ± 8.7 MPa), it falls in the range of values obtained for lumbar discs.

It is well known that crystal violet staining allows for cells visualization. In particular, microscopy and crystal violet assay provided information on the presence of the cell-laden gel into the morphologically-controlled PCL scaffold (Figures 6).

Figure 6. Microscopy and crystal violet assay at 5 days after cell seeding. Images of cell-laden multiphasic nucleus/annulus scaffolds, showing nucleus and annulus regions. Scale Bars: 200 μm.

Figure 7 reports the results obtained from the Alamar Blue assay. The number of viable cells is related to the magnitude of dye reduction and may be expressed as a percentage of Alamar Blue reduction. Specifically, a higher reduction rate indicates a higher number of viable cells.

Figure 7. Percentage of Alamar Blue reduction as a function of time. Data are reported as mean value and error bar represents the standard deviation. $^{**}p<0.01$ (p=0.0010) indicates statistically significant differences.

Regarding the statistical analysis on the percentage of Alamar Blue reduction (A-day 1, B-day 3 and C-day 7), the p-value corresponding to the F-statistic of one-way ANOVA was lower than 0.05 and 0.01, evidencing that the one or more treatments were significantly different. For this reason, Tukey's multiple comparison tests were carried out to identify which of the pairs of treatments were significantly different from each other.

The obtained results confirmed that the percentage of Alamar Blue reduction significantly increased over time indicating that hMSCs could survive and proliferate throughout the scaffolds.

Conclusions

Despite the limitations of the current research, the following conclusions were reached:

- The current work would be the first step towards the design of customized multiphasic nucleus/annulus scaffolds for potential IVD tissue engineering, consisting of 3D printed PCL scaffolds and collagen-LMW HA-4S-StarPEG-CNPs.
- Injectability and viscoelastic properties of collagen-LMW HA-4S-StarPEG-CNPs were analyzed.
- The initial J-shaped stress-strain curve of natural IVDs was reproduced and a compressive modulus of 86.3 ± 4.0 MPa, which falls in the range of lumbar discs, was achieved.
- Preliminary biological results in terms of percentage of Alamar Blue reduction as function of time also evidenced that cells were viable over the culture period.

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