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Effects of different fibre alignments and bioactive coatings on mesenchymal stem/stromal cell adhesion and proliferation in poly (ε-caprolactone) scaffolds towards cartilage repair

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Abstract

In this work, 3D biodegradable poly (ε-caprolactone) scaffolds with high porosity and interconnectivity were produced by extrusion and characterized in terms of their structural and mechanical properties. The effects of scaffold fibre alignment (0-45º and 0-90º) and of different adhesive coatings (Fibronectin, Gelatin and commercially available CELLstart™) on bone marrow mesenchymal stem/stromal cells adhesion, migration and proliferation were evaluated using AlamarBlue™ assay. Fibronectin and CELLstart™ coated poly (ε-caprolactone) scaffolds presented an improvement in cell adhesion of approximately 2-fold relatively to the non-treated scaffolds, independently of fibre alignment. Overall, all the conditions studied promoted the growth and migration of bone marrow mesenchymal stem cells in the fabricated poly(ε-caprolactone) scaffolds, without affecting their chondrogenic differentiation and extracellular matrix deposition evaluated by Alcian Blue staining.

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Keywords: Biomanufacturing; Cartilage Tissue Engineering; Cell Adhesion; Mesenchymal Stem/Stromal Cells; Poly (ε-caprolactone) Scaffolds.

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1. Introduction

As a result of the lack of effective treatments for cartilage lesions caused by trauma or by the action of degenerative diseases such as osteoarthritis and rheumatoid arthritis, Tissue Engineering (TE) strategies combining cells and biomaterial scaffolds, are gaining notoriety as they carry the promise of generating mature and functional tissue with appropriate structure and mechanical properties [1]. In terms of cell source, stem cells, particularly mesenchymal stem/stromal cells (MSC) have been widely used in cartilage TE strategies [2,3]. MSC are adult multipotent cells capable of differentiate towards osteogenic, adipogenic and chondrogenic lieneages and highly available as they can be isolated from several tissues including bone marrow (BM), adipose tissue, umbilical cord matrix and synovium [3-5]. Additionally, these cells present a high *in vitro* expansion ability, are low immunogenic and possess trophic and immunomodulatory properties that favour a regenerative microenvironment [6].

The emergence of Additive Manufacturing (AM) techniques, such as fused deposition modeling (FDM)/melt extrusion, a technique used in this work, was quite important to foster many successful developments in regenerative medicine field, particularly for cartilage TE. AM techniques offered the possibility of producing tailor-made scaffolds in a rapid and controlled manner with the desired size, shape and architecture to completely fit in the patient's defect site [7]. The application of AM techniques to TE approaches mainly consists in acquiring data of the anatomical structure that needs repair to generate a 3D CAD model of both the anatomical structure and of the biomaterial scaffold designed to be placed in patient's defect site. Then, the scaffold is manufactured and seeded with cells, and afterwards implanted into the patient to promote tissue regeneration [8]. FDM or melt extrusion is a commercially available AM technique, in which thin thermoplastic filaments or granules are melted by heating and guided by a robotic device with computer-controlled motion, to generate the 3D object. In this process, the material leaves the extruder in a hot liquid form and solidifies immediately upon cooling on the top of the previously formed layer. Therefore, each layer works as substrate for the next layer and must be maintained at a temperature just below the solidification point of the thermoplastic material to assure good interlayer adhesion [7,9]. AM technologies often use synthetic polymeric materials, such as poly (ε-caprolactone) (PCL), which was used in this study. PCL is a linear aliphatic polyester with a low melting point (≈60ºC) and a high thermal stability over 350ºC [10]. Therefore, this thermoplastic polymer is easy to process, chemically versatile and structurally stable, reasons that make PCL a suitable material for the manufacturing of 3D TE scaffolds with a highly interconnected pore network. Additionally, PCL is also a biodegradable and biocompatible material, previously approved by the FDA for the production of *in vivo* medical and drug carrier devices [12,13].

Cell adhesion to the biomaterial scaffold structure is the initial requirement for any TE strategy [14]. However, as AM techniques usually employ the use of synthetic materials lacking bioadhesive sites, lower cell attachments may occur and compromise the success of the procedure. Several approaches have been used to enhance cell adhesion to PCL scaffolds, including changing scaffold porosity [15] and pore size [16,17], controlling fiber orientation [18] and using surface modification approaches such as coating the scaffold structure with extracellular matrix (ECM) molecules containing cell-binding motifs [19-21]. On the other hand, chondrogenic differentiation requires MSC condensation, which can be inhibit by materials that promote too much cell stretching upon their adhesion to scaffolds, implying that a compromise between cell adhesion and condensation is required for TE cartilage repair strategies [22]. The objective of this study was to preliminary assess the effect of different PCL scaffold fibre alignments (0-45º/0-90º) and of different standard cell culture bioadhesive coatings in promoting BM MSC adhesion and proliferation towards the development of an optimized and integrated TE strategy for cartilage regeneration.

2. Materials and Methods

2.1. PCL scaffold fabrication and characterization

PCL (MW 50.000 Da, Sigma-Aldrich) scaffolds with both fibre orientations (0-45º and 0-90º) were produced by FDM using a Bioextruder machine as previously described [23,24]. The scaffolds were then structurally characterized by scanning electron microscopy (SEM, Hitachi model S2400) and by micro-computed tomography analysis (μ-CT, Scansky 1174v2, Brucker version 1.1). For SEM analysis, scaffold samples were coated with a 45 nm gold/palladium layer using a sputter coater (Quorum Technologies model E5100). PCL scaffolds of both architectures with dimensions 5mm x 5mm x 3mm were also assessed in terms of their mechanical properties under

compressive testing using an Instron (model 5544) machine equipped with a 2 kN load cell and a 50 mm diameter cylindrical compression plate and operating with an extension rate of 1 mm/min. For each scaffold configuration, 5 scaffold samples were tested. The results of the tests were then analyzed using the Bluehill® 3 software. The Young's/compressive modulus of elasticity was calculated by the slope of the initial linear region of the stress-strain curve, in which the compressive stress is defined as the compressive load per unit area of the minimal original cross section carried by the test specimen at any given moment and the compressive strain corresponds to the change in length per unit of original length along the longitudinal axis.

2.2. PCL scaffold coating and wettability/contact angle assessment

PCL scaffolds of both configurations were firstly sterilized (12 hours UV treatment and ethanol 70% (v/v, Merck) washing) and then submitted to the different surface modification protocols by completely submersing the samples in the respective adhesive coating solutions: (i) human-derived Fibronectin 5 µg/mL (Sigma-Aldrich) solution in Phosphate buffered saline (PBS, Gibco) for 1 hour at 37ºC; (ii) Gelatin 0.2% (Sigma-Aldrich) solution in PBS for 1 hour at 37ºC; and (iii) CELLstart™ 1:200 (Life Technologies) solution in PBS for 1 hour at 37ºC). The scaffold samples were coated right before the cell culture studies or otherwise maintained at 4ºC until usage. Wettability was measured by the contact angle, which is defined by the intersection of the liquid-solid interface. When the contact angle is lower than 90º the material is considered hydrophilic while above 90º is hydrophobic. To study the effect of the different surface coatings on PCL's wettability, films were produced by dissolving PCL in chloroform (Merck) and by promoting solvent evaporation overnight inside a chemical flow hood. Afterwards, PCL films were treated with the different coating solutions and the contact angle was measured using a DSA25B goniometer (Krüss) at 2 different time points (t=0 sec and t=30 sec). For that, a sessile drop of distilled water was added on the top of the films and the results were analyzed using the software Drop Shape Analysis 4 version 2.1. For each condition, 5 measurements were performed.

2.3. BM MSC seeding and culture in PCL scaffolds

For the *in vitro* cell culture studies, $6x10^4$ human bone marrow-derived (BM) MSC (passage 5) were seeded on the top of each sterile and coated PCL scaffold (5mm x 5mm x 3mm), which were previously placed in a 24-well ultra-low attachment plate (VWR). Cells were left to incubate at 37ºC/5% CO_2 for 90 min in order to promote cell adhesion. Afterwards, culture media was added to completely immerse the scaffold. These experiments were composed by 2 phases: an expansion phase during the first 2 weeks followed by a chondrogenic differentiation phase for 3 weeks. During the expansion stage, scaffolds were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) with 10% fetal bovine serum (FBS, Life Technologies), and 1% antibiotic-antimycotic (anti-anti, Gibco) solution, while during chondrogenesis, scaffold samples were cultured using the StemPro Chondrogenesis Differentiation kit (Gibco) and 1% anti-anti solution. Throughout all the experiment, medium was fully replaced each 3-4 days and all samples were cultured inside an incubator under hypoxic conditions (37ºC/5% CO_2 and 5% O_2) to promote MSC chondrogenic differentiation.

2.4. Evaluation of BM MSC adhesion, proliferation and chondrogenesis

Cell adhesion (Day 1 after seeding) and cell proliferation (Days 1, 7, 14, 21, 28 and 35) were assessed using the AlamarBlue™ indirect cell quantification assay (AB, Invitrogen), following the manufacturer's guidelines. Briefly, scaffold samples were incubated 2h30min in an AB solution (1:10 diluted in culture media), and afterwards, the fluorescence intensity was measured in a multiplate fluorometer (Infinite 200 PRO, TECAN) with an excitation wavelength of 560 nm and an emission wavelength of 590 nm. Cell quantification is reported as equivalent cell numbers, which were estimated through correlation between 590 nm light absorbance fluorescence intensity values with counted cells cultivated in standard tissue culture polystyrene well plates (BD falcon). Note that cell proliferation during the first day of culture could also contribute to report "cell adhesion" percentages, considering that the population doubling time for MSC was observed to occur at values higher than 24 hours [26].

At the end of the experiment (Day 35), PCL scaffolds were dehydrated using an ethanol gradient solution treatment (20%/40%/60%/90% and 96% v/v for 30 min each) and analyzed by SEM as described above. Additionally, the chondrogenesis of BM MSC in the different conditions was assessed by Alcian Blue (Sigma-Aldrich) staining, which labels sulfated glycosaminoglycans (sGAG). For that, samples were washed once with PBS, fixed with 2% PFA for 20 min and incubated with a 1% Alcian Blue solution (in 0.1N HCl) for 1 hour. Afterwards, scaffolds were rinsed twice with PBS, washed once with distilled water and observed under a light microscope (LEICA® DMI3000B).

2.5. Statistical analysis

Data are presented as mean ± standard deviation (SD). The statistical analysis was performed using the analytical features of GraphPad Prism 7. Statistical significant differences between two independent sets of samples (conditions) were assessed by an unpaired t-test, in which p-value represents the probability that the null hypothesis is true, in the particular case the null hypothesis is the values compared to be similar to each other, considering that measured values follow a Gaussian distribution. Lower p-values indicate a lower interception of the Gaussian distributions and thus reject the null hypothesis of the values to be similar in favor of the alternative hypothesis, i.e. the values to be different of each other. In this work, statistically different values were considered for p-value < 0.05 (*p <0.05, **p <0.01 and ***p <0.001). For the *in vitro* cell culture studies, triplicates of each condition were used (same donor). In the mechanical testing and contact angle measurements, 5 samples of each condition were used.

3. Results and Discussion

PCL scaffolds with different fibre alignments (0-45º/0-90º) were fabricated by extrusion and their structure was characterized by SEM (Fig. 1.) and by µ-CT (Fig. 2.). Using µ-CT analysis, important scaffold features such as porosity, interconnectivity and surface area to volume ratio were estimated (Table 1.). Both scaffold configurations presented high interconnectivities, which is of great importance for good cell's nutrient supply and waste removal in a TE strategy.

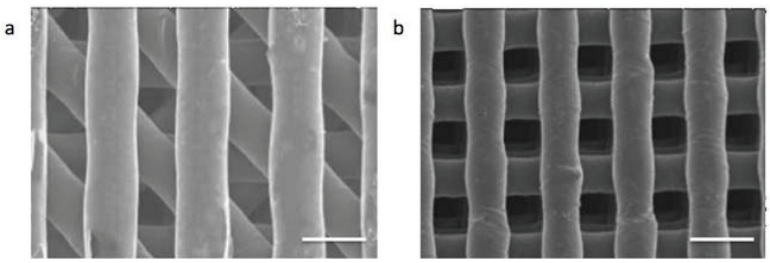

Fig. 1. SEM images of PCL scaffolds with (a) 0-45º and (b) 0-90º fibre alignments (Scale bar 500 µm).

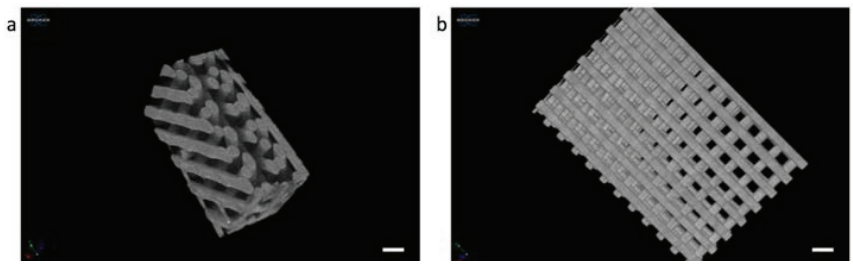

Fig. 2. µ-CT analysis of PCL scaffolds with (a) 0-45º and (b) 0-90º fibre orientation (Scale bar 500 µm).

Table 1. Characteristics of PCL scaffolds estimated by μ-CT analysis.

Scaffold features	0-45º	0-90º
Porosity (%)	52.4	56.6
Surface area to volume ratio (mm^{-1})	22.7	21.2
Interconnectivity (%)	98.8	99.7

The manufactured scaffolds were also characterized in terms of their mechanical properties (Young's compressive modulus) under compressive testing in the apparatus shown in Fig. 3a. As represented in Fig. 3b, PCL scaffolds with 0-45º angles between fibers present slightly higher compressive modulus (35 ± 3 MPa) compared to 0-90º PCL scaffolds (30 ± 1 MPa), which may be explained by their lower porosity.

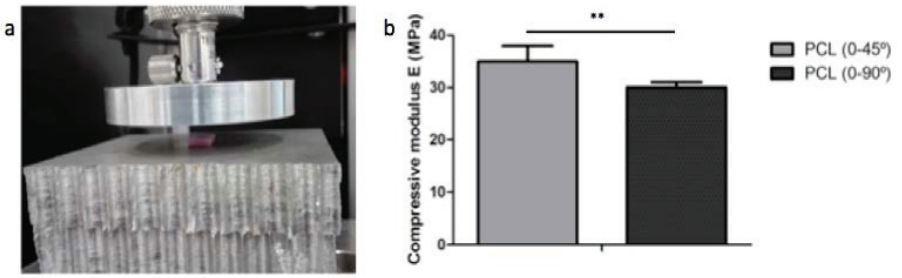

Fig. 3. Compressive mechanical testing of PCL scaffolds: (a) experimental apparatus in an Instron equipment and (b) compressive modulus values for both configurations of PCL scaffolds (0-45º/0-90º); N=5 scaffold samples; ** p<0.01.

The effect of the different surface coatings on PCL material wettability was studied by measuring the water contact angle in the PCL films (Fig. 4). The untreated PCL films presented a contact angle superior to 90º, which corresponds to a hydrophobic behavior as previously reported in the literature [24]. After treatment with the different bioadhesive coatings, all the conditions promoted a decrease in the contact angle for values lower than 90º, making the scaffold surface hydrophilic, which may lead to higher cell adhesions. This effect was significantly more evident when Fibronectin and CELLstart™ coatings were used.

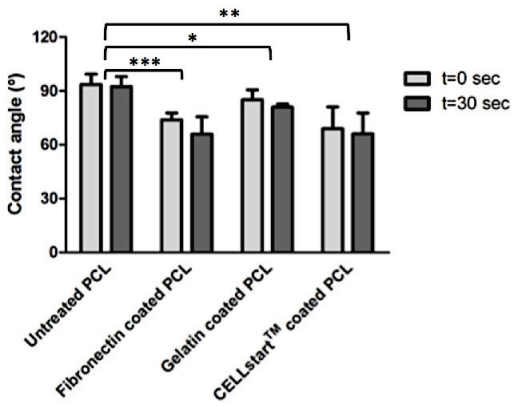

Fig. 4. Contact angle values for the different surface coatings studied; N=5 samples; * p<0.05, ** p<0.01, *** p<0.001 relative to untreated PCL.

The percentage of BM MSC adhesion (Fig. 5) to both PCL scaffold configurations treated with the different surface coatings was evaluated at day 1 by calculating the ratio between the number of viable equivalent cells in the scaffold (estimated indirectly using the AlamarBlue™ assay) and the number of cells seeded at the beginning of the experiment. Fibronectin and CELLstart™ treatments promoted a statistically significant improvement in cell adhesion of approximately 2-fold relatively to the untreated PCL samples for both fibre alignments. However, when the Gelatin coating was used no significant improvement was noticed. These findings are coherent with the results shown in Fig. 4, as lower contact angles (more hydrophilic material) correspond to higher cell adhesions. Moreover, no significant differences in cell adhesion were observed between scaffolds with different fibre alignments.

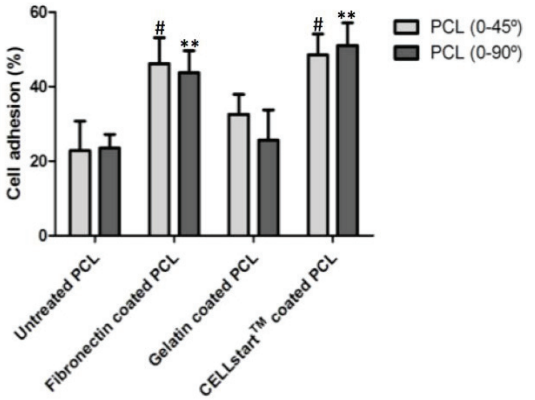

Fig. 5. BM MSC adhesion to PCL scaffolds treated with different surface coatings; N=3 samples from the same donor; ** $p<0.01$, relative to untreated PCL (0-90º) and # $p<0.05$ relative to untreated PCL (0-45º).

Concerning the cell proliferation studies (Fig. 6), all the conditions tested supported BM MSC growth throughout the culture period. In the case of the 0-45º PCL scaffolds (Fig. 6a), no relevant differences were observed in the final cell numbers for the different surface coatings tested. Regarding the 0-90º PCL scaffolds (Fig. 6b), Fibronectin coated samples presented a noticeable higher final number of cells per scaffold ($1.20 \times 10^5 \pm 2.79 \times 10^3$) compared to the remaining conditions. As it is possible to observe in Fig. 5 and Fig. 6, higher initial cell adhesions did not result necessarily in higher final equivalent cell numbers at the end of the culture. An explanation for this may be related with the long culture period employed in this work (35 days), in which the BM MSC were capable of recover from lower cell attachments and proliferate to populate all the scaffolds structure. However, for shorter *ex-vivo* culture periods, a high initial cell adhesion to the scaffold structure should be crucial for the success of the TE strategy.

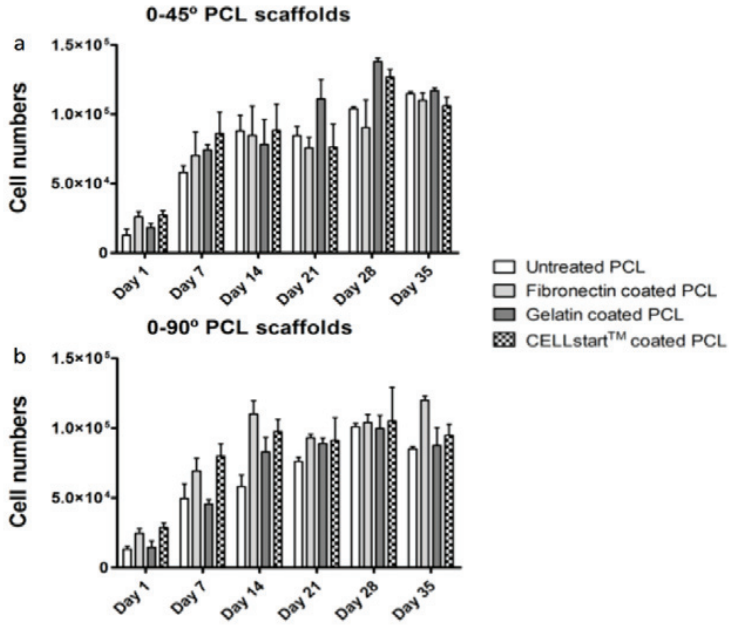

Fig. 6. BM MSC proliferation in (a) 0-45º and (b) 0-90º PCL scaffolds treated with different adhesive coatings. N=3 samples from the same donor.

At the end of the experiment, all BM MSC-PCL scaffold tissue-constructs were imaged by SEM (Fig. 7) and assessed for chondrogenesis using the Alcian Blue staining protocol (Fig. 8). As it is possible to observe in Fig. 8, all the conditions stained positive for sGAG, major components of the cartilage tissue. Further studies such as qRT-PCR analysis of chondrogenic gene markers and histological/immunohistochemistry assessment of the final tissue-constructs obtained will be required for a more complete chondrogenesis characterization.

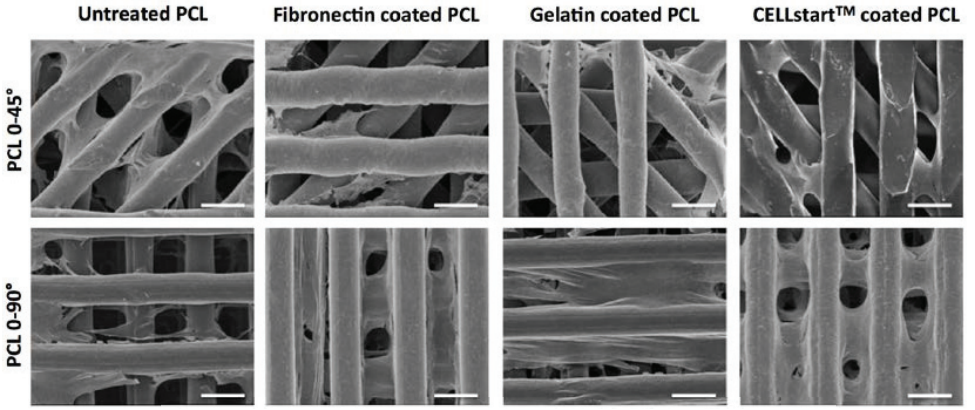

Fig. 7. SEM analysis of the BM MSC – PCL scaffold tissue constructs (different fibre alignments and surface coatings) at the end of the experiment (Scale bar 500 μm).

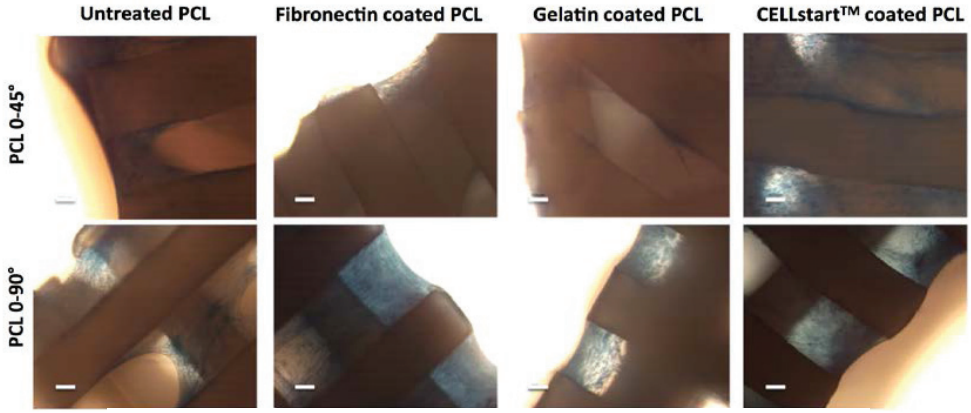

Fig. 8. Alcian Blue staining of the BM MSC – PCL scaffold tissue constructs (different fibre alignments and surface coatings) at the end of the experiment (Scale bar 100 μm).

4. Conclusions

In conclusion, 3D highly interconnected PCL scaffolds with two different fibre alignments (0-45º and 0-90º) were successfully fabricated and characterized in terms of their structural and mechanical properties. With the objective of enhancing BM MSC adhesion to the produced PCL scaffolds, three different standard coatings containing adhesive proteins/motifs were tested. Cell adhesion to PCL scaffolds was increased by approximately 2-fold relatively to the untreated condition by using Fibronectin and CELLstart™ coating protocols, independently of the scaffold architecture. These results seemed to be correlated with the ones obtained after contact angle assessment of the coated and untreated PCL material, as more hydrophilic samples (lower contact angle) correspond to higher cell adhesions in the *in vitro* culture studies. However, such correlation between improved adhesion and lower contact angle was not observed for gelatin coating suggesting that specificity of the biological motives also play a role in mediating cell adhesion. Despite the differences observed in the initial cell adhesion, all the conditions tested supported BM MSC proliferation, migration and chondrogenic differentiation.

Overall, our results provide some initial insights for the development of a procedure to enhance BM MSC adhesion to PCL scaffolds towards an integrated and optimized strategy for cartilage TE.

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