Microfabrication of a biomimetic arcade-like electrospun scaffold for cartilage tissue engineering applications

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Abstract

Designing and fabricating hierarchical geometries for tissue engineering (TE) applications is the major challenge and also the biggest opportunity of regenerative medicine in recent years, being the in vitro recreation of the arcade-like cartilaginous tissue one of the most critical examples due to the current inefficient standard medical procedures and the lack of fabrication techniques capable of building scaffolds with the required architecture in a cost and time effective way. Taking this into account, we suggest a feasible and accurate methodology that uses a sequential adaptation of an electrospinning-electrospraying set up to construct a system comprising both fibres and sacrificial microparticles. Polycaprolactone (PCL) and polyethylene glycol were respectively used as bulk and sacrificial biomaterials, leading to a bi-layered PCL scaffold which presented not only a depth-dependent fibre orientation similar to natural cartilage, but also mechanical features and porosity compatible with cartilage TE approaches. In fact, cell viability studies confirmed the biocompatibility of the scaffold and its ability to guarantee suitable cell adhesion, proliferation and migration throughout the 3D anisotropic fibrous network. Additionally, likewise the natural anisotropic cartilage, the PCL scaffold was capable of inducing oriented cell-material interactions since the morphology, alignment and density of the chondrocytes changed relatively to the specific topographic cues of each electrospun layer.

Keywords: Cartilage tissue engineering, arcade-like, electrospinning, 3D fibrous scaffolds
1. Introduction

One key design criteria of the next generation tissue engineering (TE) scaffolds is the accurate recreation of the 3D hierarchical complexity of the extracellular matrices (ECMs), including the mimicking of the architectures, biochemical gradients and mechanical properties of the native tissues [1-3]. This purpose is particularly evident in cartilage TE applications [4] due to the challenges associated with the in vitro reproduction of the anisotropic arrangement of the cartilaginous collagen network, where the orientation of the fibrils progress from parallel to the subchondral bone surface in the superficial zone, to random in the middle zone, to perpendicular in the deepest region. The successful replication of these depth-dependent topographic cues is mandatory to modulate an enhanced cell response since the chondrocytes are capable of adapting their morphology and biochemical expression patterns according to the geometry of the surrounding microenvironment [5]. Indeed, considering that the natural regeneration process of articular cartilage is impaired by the combination of intrinsic factors such as low cell density, poor chondrocyte proliferation and absence of vascularization, a clinically fitted biomimetic scaffold could be a crucial tool to replace the damaged cartilaginous tissue and consequently restore the functionality of the injured area [6].

In this regard, during the past few years, a very impressive set of advanced scaffolds has been reported, presenting not only promising results regarding chondrogenesis, but also pointing out complementary cartilage TE parameters like bioactivation pathways [7], cell culture conditions [8] and, principally, groundbreaking design and fabrication methodologies [9] to build 3D cellular microenvironments. As a matter of fact, recent studies have pointed out that hydrogels loaded with stem cells can be presented in form of microgels [10] or bioinks [11] and then assembled or bioprinted, respectively, into 3D constructs capable of boosting chondrogenic differentiation. Other cartilage scaffolding approaches allow the possibility of engineering porous systems [12; 13] with anisotropic pore size and/or orientation able to mimic the depth-dependent biochemical and biomechanical properties of natural cartilage, leading to a spatially
controlled chondrocyte behaviour both *in vitro* and *in vivo*. Although hydrogels and porous scaffolds can present interesting hierarchical architectures able to stimulate cartilaginous tissue regeneration, both designs are insufficient to guarantee topographical cues similar to their natural counterparts, where the fibrous morphology and orientation of the cartilage ECM are crucial to ensure structure and function. Thus, fibrous scaffolds have emerged as a preponderant option for cartilage TE, bringing electrospinning to a frontrunner position relatively to other microfabrication techniques (e.g. self-assembly and phase separation) due to its cost-effectiveness, reproducibility and versatility regarding important designing fundamentals such as fibre diameter, orientation and chemical composition [14]. However, since the conventional electrospinning is usually limited to the producing of 2D dense fibrous meshes with an orientation dependent on the used collector, there has been a growing focusing to adjust this technology to match the requirements of a biomimetic 3D fibrous scaffold [9; 15] for cartilage TE applications. For example, by combining optimal technological parameters (e.g. solution viscosity, voltage, working distance and ambient conditions) with a predefined XY translation of the collector, Chen et al. [16] were able to use a writing electrospinning modality to accurately fabricate an ultrathin cartilage mimetic scaffold capable of enhancing chondrocyte differentiation and directing the new tissue according to each simulated cartilaginous fibrous zone. In an analogue approach, a 3D fibrous/porous network was fabricated via melt electrospinning and then incorporated into a soft hydrogel as reinforcing agent with the purpose of ensuring suitable anisotropic biomechanical properties for neocartilage formation [17]. Alternatively, using multi-layer electrospinning do not require complex equipment to control the collector movement, leading to a simpler but time-consuming process to build 3D fibrous systems with controllable fibre size and orientation. In fact, a recent work reported a five-layered electrospun scaffold with not only a hierarchical fibre orientation, but also with depth-dependent biological properties due to the sequential electrospinning of two types of collagen [18]. However, this type of scaffolds usually present low thickness and small pore size, limiting the success of cell infiltration and
growth. In this regard, other suggested hypotheses to fabricate implantable functional cartilage
TE scaffolds include: direct electrospinning into a 3D mould with the desired architecture [19];
engineer composite scaffolds able to intercalate layers of electrospun fibres and porous
decellularized extracellular matrix sponges [20] and sequenciate freezing, frieze-drying and
thermal treatment of an initial dispersion of electrospun fibres [21]. Taking this into account,
since there is currently no feasible and efficient methodology able to guarantee the precise
recreation of the arcade-like fibrous organization of native cartilage, we suggest a simple four-
step approach to develop a biomimetic 3D bi-layered electrospun scaffold with anisotropic
features compatible with cartilage TE protocols. Indeed, based on the obtained results, we expect
that the accessibility and scalability of the presented microfabrication technique could support
and potentiate a wide range of static and dynamic cell culture protocols concerning cartilage
regeneration.

2. Materials and methods

2.1 Materials

The Polycaprolactone (PCL) with a molecular weight of 80 000 Da, Polyethylene glycol (PEG)
with a molecular weight of 8 000 Da, 2,2,2-Trifluoroetanol (TFE), Dimethylformamide (DMF),
Chloroform, Bos taurus articular cartilage progenitor cell line (CP5), Dulbecco’s Modified
Eagle’s Medium/Nutrient Mixture F-12 Ham (DME/F-12, 1:1 mixture), Penicillin-Streptomycin
(Pen-Strep), Fetal Bovine Serum (FBS), Phosphate-Buffered Saline (PBS), Resazurin sodium
salt powder and 4’,6-diamidino-2-phenylinodole DAPI were purchased from Sigma Aldrich. The
Formaldehyde was purchased from ACROS Organics. The Triton X-100 was purchased from
Fisher Scientific. The Phalloidin-iFluor 488 was purchased from ABCAM.

2.2 Microfabrication of the bi-layered PCL electrospun scaffold
The PCL and PEG solutions were prepared by dissolving the polymers in a mixture of TFE:DMF (75:25, v:v) with a final concentration of 12% w/v and in chloroform with a concentration of 1.5 g mL\(^{-1}\), respectively. The PCL solution was stirred at room temperature overnight while the PEG solution was prepared 45 minutes before the electrospinning process, including a 30 minutes period of stirring at a temperature of 50 °C followed by a cooling period at room temperature. Figure 1 summarizes the microfabrication procedure. Firstly (STEP 1 of Figure 1), the PCL and PEG solutions were simultaneously electrospun and electrosprayed using an applied voltage of 25 kV, distinct flow rates (1 mL h\(^{-1}\) for PCL and 4.5 mL h\(^{-1}\) for PEG) and different needle tips (21G for PCL and 18G for PEG). Both the PCL microfibres and the PEG microparticles were collected in a rotating drum (width = 200 mm; diameter = 200 mm; 750 RPM) using a working distance of 15 cm. The final mesh presented a thickness of approximately 300 μm. A small rectangle (length = 4 mm and width = 3 mm) was cut from the PCL-PEG mesh and then rolled up inside a teflon mould to form the spiral shaped cylinder with defined dimensions (diameter = 3 mm and high = 3 mm) that is illustrated in the STEP 2 of Figure 1. This spiralled cylinder was used as Bottom Layer (BL) for the final scaffold since it presented vertically aligned electrospun fibres relatively to the Top Layer (TL), which was fabricated according to STEP 3 of the Figure 1. In this phase, the rotating drum was replaced by a static collector able to support the BL, making it a target for the newly formed PCL-PEG network with a thickness of approximately 500 μm. The bi-layered PCL-PEG scaffold was finally subjected to a series of washes with distilled water at 37 °C for 2 weeks in order to remove the sacrificial PEG microparticles and consequently optimize the inter-fibre distance (STEP 4 of Figure 1). The final anisotropic electrospun 3D structure was denominated PCL scaffold and presented final dimensions (thickness = approximately 3.5 mm; diameter = 3 mm) that are compatible with the native articular cartilaginous tissue (2 mm to 4 mm of thickness [22]).

2.3 Characterization of the bi-layered PCL electrospun scaffold
2.3.1 Morphological characterization. A scanning electron microscope (SEM) Hitachi SU 70 (Hitachi High-Technologies) was used to evaluate the topographic features of the PCL scaffold. More precisely, the diameter and fibre-fibre distance together with the dimensions of the sacrificial microparticles were studied by directly analysing ten SEM pictures of the PCL-PEG network before and after the washing procedure described in the STEP 4 of Figure 1. Similarly, a SEM analysis was conducted to investigate the anisotropic morphology of the PCL scaffold.

2.3.2 Mechanical characterization. Before the mechanical analysis, the swelling properties of the PCL scaffold were evaluated by immersing the samples (n = 5) into distilled water for 72 hours at room temperature. The variation of the swelling ratio was calculated for different time intervals using the following formula:

\[ R = \frac{(W_s - W_d)}{W_d} \]

where R is the swelling ratio (mg mg\(^{-1}\)), the \( W_s \) is the weight of the swollen scaffold and \( W_d \) the weight of the dried scaffold.

The swollen PCL scaffolds were then subjected to compressive tests using a Shimadzu MMT-101 N (Shimadzu Scientific Instruments) with a load cell of 100 N. The compressive moduli of the samples were calculated by analysing the slope of the stress-strain curves in the linear region after a pre-charge of 0.07 N. The samples were compressed until 15 % strain with a compressive rate of 0.5 mm min\(^{-1}\).

2.3.3 Biocompatibility studies. Prior to cell culture, the PCL scaffolds were sterilized in 70% ethanol for 3 hours and then carefully washed with PBS before incubation in DMEM/F-12 medium for 1 hour. Each scaffold was placed in a well of a 96-well plate and seeded with 0.5 x 10\(^6\) CP5 cells suspended in 50 μL of culture media. The cell adhesion was guaranteed by incubating the PCL scaffolds for 2 hours at 37 ºC and 5% CO\(_2\). Afterwards, fresh medium was added until a final volume of 200 μL per well was reached. The cell viability was evaluated via a non-toxic resazurin metabolic assay [23; 24]. Briefly, at specific time points (day 1, 4, 7, 14
and 21) the scaffolds were incubated with fresh medium containing 10% of a resazurin solution (0.1 mg mL\(^{-1}\) in PBS) during a period of 4 hours. Then the resazurin reduction to resorufin was determined by spectrophotometry (Synergy\(^{TM}\) HTX), more precisely, the absorbances at 570 nm and 600 nm were measured since these are the resazurin maximum absorbance and the resorufin maximum absorbance, respectively. The final optical density value (O.D.) was calculated with the following equation:

\[
OD = \text{sample} \left( \frac{OD_{570 \text{ nm}}}{OD_{600 \text{ nm}}} \right) - \text{negative control} \left( \frac{OD_{570 \text{ nm}}}{OD_{600 \text{ nm}}} \right)
\]

where the negative control was a scaffold without cells. Then, the cell viability was calculated with the formula:

\[
\text{Cell viability (\%) = } \frac{OD}{OD_{\text{control}}} \times 100
\]

where the OD control was the O.D. calculated for the cells seeded onto a culture well.

The cell attachment and morphology were evaluated via fluorescence and SEM microscopy after 21 days. Before the cytochemical staining of the actin filaments and nuclei, the cells were fixed in a 4% paraformaldehyde solution for 30 minutes and then permeabilized using 0.1% v/v Triton X-100 for 5 minutes. After carefully washed with PBS, the scaffolds were fluorescently stained in a dark room for F-actin (Phalloidin-iFluor 488) and for nuclei (DAPI) during periods (intercalated with PBS washes) of 90 and 15 minutes, correspondingly. To visualize the cells a fluorescence microscope (Axioimager M2, Zeiss) with magnification of 10x/0.25 was used. Complementary, additional scaffolds with fixed cells were preliminarily dehydrated in 50%, 70%, 90%, 95% and 100% ethanol for 20 minutes each. Then, the samples were sequentially dried with hexamethyldisilazane:ethanol solutions with ratios 1:2; 2:1 and 100% hexamethyldisilazane before SEM observation (Hitachi TM 4000 plus - Hitachi High-Technologies).

3. Results and discussion
PCL was selected as bulk biomaterial for the microfabrication of the bi-layered anisotropic scaffold due to its well-known set of properties [25], particularly the remarkable biocompatibility/biodegradability and the versatile manipulation/stability features that allow an easy shaping towards dissimilar TE scaffolds (e.g. 2D films, electrospun fibres, 3D porous networks, etc.). As it is possible to see from the STEP 4 of the Figure 1, the PCL scaffold presented a 3D arcade-like fibrous structure likewise its natural counterpart due to the perpendicularity established between the vertical alignment of the BL relatively to the horizontal orientation of the fibres located onto the TL. More precisely, this biomimetic fibre arrangement was successfully accomplished via a sequential microfabrication process that started by simply curling a rectangle cut from the initial PCL-PEG aligned network to form a spiralled cylinder with the desired vertical fibre orientation. Afterwards, as the initial rotating drum was replaced by a static collector, it was possible to target the BL with the intention of fabricating a new fibre-microparticle system above it and, consequently, ensuring an orientation of 90° with respect to the deepest zone. For both TL and BL, PCL was electrospun simultaneously with the electrospraying of PEG with the purpose of, firstly, building a network of both fibres and sacrificial microparticles and, secondly, enabling the enlargement of the fibre-fibre distance after PEG removal. This tactic to increase the pore size of the PCL electrospun mesh is a simple variation of other reported studies [26; 27] in which the authors used the leaching of water-soluble materials located into the fibrous networks to enhance their porosity. Indeed, it was possible to optimize the inter-fibre distance without damaging the integrity of the 3D PCL scaffold nor disrupting the fibre morphology of both layers, where the fibres presented a diameter of 1.47 ± 0.50 μm and maintained their depth-dependent orientation together with a smooth and defect free surface. Figure 2a compares the pore size distribution before and after the removal of the electrosprayed PEG microparticles (diameter = 17.19 ± 9.41 μm), showing an increasing of 15% and 11% in the number of pores with dimensions between 10 and 20 μm and superior to 20 μm, respectively. Accordingly, the initial number of pores with smaller dimensions (< 10 μm)
was successfully reduced from 63% to 30%. It is expected that this upgrading on the fibre-fibre
distance could not only encourage cell proliferation and 3D infiltration into the PCL scaffold [28; 29], but also enhance the nutrient/waste diffusion across its bi-layered fibrous network. In
particular, the effects of the pore size increment will complement the intrinsic advantages of the
spiral shaped scaffolding design adopted for the BL (STEP 3 of Figure 1) because the gaps
between the concentric walls are easily capable of boosting 3D cell migration, oxygen and
nutrient supply and metabolic waste removal [30; 31].

The integrity of the PCL scaffold was confirmed by studying its mechanical response under
compressive loading at low strains (Figure 2b) and its swelling properties (Figure 2c). As
anticipated, the stress-stain curve presented a linear regime, indicating no considerable buckling,
collapsing or densification of the 3D anisotropic fibrous network for the applied compressive
regime. The calculated compression modulus was 38.84 ± 2.20 kPa, which is a value compatible
with cartilage TE applications [4; 11; 32] considering that it matches the range of values of the
native cartilaginous tissue (89.5 ± 48.6 kPa [33]) albeit the considerable variations not only due
to its hierarchical anatomy and physiology, but also because of the origin of the tissue and the
conditions of the experiment. The mechanical tests were performed with swollen PCL scaffolds
in order to guarantee the influence of the water uptake capacity during the restoration of their
original shape after compression, similarly to the natural response of the cartilaginous ECM [4; 5; 21]. It is important to notice the success of the presented methodology to build robust bi-
layered anisotropic fibrous systems since neither the mechanical nor the swelling testing have
compromised the attachment between the TL and the BL, ensuring the stability of the initial
geometric and morphologic features of the PCL scaffold.

The cell viability was analysed via a non-destructive metabolic resazurin assay and, as it is
possible to see from Figure 3a, the PCL scaffold remained biologically viable for the culture
period of 21 days. Adding to the excellent levels of biocompatibility, the anisotropic electrospun
network proved to be suitable for an enhanced cellular proliferation since there was a steadily
increasing number of viable cells over 7 days followed by a plateau that was maintained until the end of the culture. In fact, microscope analysis confirmed that cells were able to successfully perform an efficient 3D migration across the scaffold by covering the entire surface of both TL and BL. In detail, fluorescence images of a longitudinal cross section of the PCL scaffold (Figures 3b and 3c) shows a uniform cellular distribution throughout the 3D anisotropic fibrous network, which was a direct result of an enhanced cell adhesion process onto the electrospun fibres, a quick proliferation ratio (during the first 7 days) and the maintenance of optimized nutrient and oxygen supply to the cells. Another expected effect of improving the pore size and interconnectivity due to the removal of the PEG microparticles was a superior cell infiltration through the TL (Figure 3d) and the subsequent enabling of their passage from the superficial region of the scaffold to the deepest layer, where the spiralled geometry boosted the 3D cell migration. Indeed, analogously to other reports [30; 31; 34; 35], the gaps between the concentric fibrous walls of the BL were able to facilitate the adequate nutrient transport and metabolic waste removal across the system, leading consequently to an enhanced cell growth and a preferential longitudinal proliferation (Figure 3e). Furthermore, the distinct effects on the cell morphology induced by the complementary topographic cues of both TL and BL were studied via SEM analysis. The vertical orientation of the electrospun spiral branches relatively to the TL fibrous network (Figure 3f) was capable of promoting chondrocyte elongation along the fibres with the cells showing lamellipodia and filopodia-like extensions typical of spreading cells (Figures 3g and 3h) [36]. Another interesting detail of the 3D migration towards the BL is illustrated in Figure 3i, where it is possible to observe that the CP5 chondrocytes were able to bridge two adjacent fibrous walls of the electrospun spiral. From the top view of the PCL scaffold showed in Figure 3j is evidenced not only the contrast between the geometries of both layers, but also the dense cell layer that covers the TL. As a matter of fact, contrary to the cells located onto the BL, the CP5 chondrocytes attached to the superficial zone of the PCL scaffold showed a flattened polygonal morphology (Figures 3k and 3m) characteristic of the 2D electrospun scaffolds [37].
Overall, the presented biocompatibility results, although preliminary since it is necessary further characterization of the cell behaviour by evaluating the gene expression and the newly produced ECM, are able to confirm the capability of the PCL scaffold to mimic major depth dependent features of the natural cartilaginous tissue [4] such as cell morphology (flattened and elongated shaped chondrocytes onto the TL and BL, respectively), higher cell density in the superficial region and oriented cell-material interactions (progressing from horizontally aligned in the TL to a vertically arrangement in the BL).

4. Conclusion

In this work, we presented a new methodology for recreating in vitro the arcade-like topography of the cartilaginous tissue, where the orientation of the fibres progress from vertical in the deepest zone to horizontal in the superficial zone. Indeed, by sequentially changing the simultaneous electrospinning-electrospraying set up, it was possible to engineer a hierarchical bi-layered PCL scaffold with mechanical properties and pore interconnectivity suitable for cell culture protocols. Additionally, the depth-dependent orientation of the 3D fibrous network was capable of influence the morphology, alignment and density of the cultured chondrocytes likewise its natural counterpart. Thus, it was possible to validate an original and simple microfabrication process able to build 3D biomimetic fibrous architectures compatible with cartilage TE applications. Future work will include a more detailed characterization of the cell response and its modulation by mechanical stimuli.

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