Author Manuscript

Published in final edited form as:

Journal of Biomaterials Applications

Vol. 0(0) 1 – 12, 2021

DOI: 10.1177/08853282211064403

https://doi.org/10.1177/08853282211064403

Title:

MULTI–LAYERED ELECTROSPINNING AND ELECTROSPRAYING APPROACH: EFFECT OF POLYMERIC SUPPLEMENTS ON CHONDROCYTE SUSPENSION

A. Semitela[1], A. Pereira[1], C. Sousa[2], A. F. Mendes[2], PA.A.P. Marques[1], A. Completo[1]*.

[1] Department of Mechanical Engineering, University of Aveiro, Portugal

[2] Centre for Neuroscience and Cell Biology, University of Coimbra, Portugal

*** Corresponding Author:**

E-mail addresses: completo@ua.pt (A. Completo)

ABSTRACT

Articular cartilage was expected to be one of the first tissues to be successfully engineered, but replicating the complex fibril architecture and the cellular distribution of the native cartilage has proven difficult. While electrospinning has been widely used to reproduce the depth–dependent fibre architecture in 3D scaffolds, the chondrocyte-controlled distribution remains an unsolved problem. To incorporate cells homogeneously through the depth of scaffolds, a combination of polymer electrospinning and cell seeding is necessary. A multi-layer approach alternating between polymer electrospinning with chondrocyte electrospraying can be a solution. Still, the success of this process is related to the survival rate of the electrosprayed chondrocytes embedded within the electrospun mesh. In this regard, the present study investigated the impact of the multi-layered process and the supplementation of the electrospray chondrocyte suspension with different concentrations of Gelatin and Alginate on the viability of electrosprayed chondrocytes embedded within a Polycaprolactone/Gelatin electrospun mesh and on the mechanical properties of the resulting meshes. The addition of Gelatin in the chondrocyte suspension did not increase significantly ($p > 0.05$) the percentage of viable electrosprayed chondrocytes (25 %), while 3 wt% Alginate addition led to a significant ($p < 0.05$) increase in chondrocyte viability (50 %) relative to the case without polymer supplement (15 %). Furthermore, the addition of both polymer supplements increased the mechanical properties of the multi–layer construct. These findings imply that this multi-layered approach can be applied to cartilage TE allowing for automated chondrocyte integration during scaffolds creation.

Keywords**:** Electrospray; Electrospinning; Chondrocyte; Gelatin; Alginate; Viability; Cartilage tissue engineering

INTRODUCTION

Cartilage tissue engineering (TE) strategies are constantly evolving in an effort to mimic the fibril architecture and cellular distribution of the native articular cartilage [1–3]. While for the recreation of the native fibril disposition substantial progress has been made through the development of 3D electrospun scaffolds with specific fibre orientations [4–7], it is still challenging to ensure a controlled chondrocyte distribution within the depth of the scaffold [8–10]. Conventional seeding techniques on 3D electrospun scaffolds usually result in poor cell infiltration throughout the scaffold due to their characteristic small pores [9–11]. So, to efficiently include chondrocytes on these structures, a reasonable option would be to directly seed the cells onto the fibres during scaffolds production, using simultaneous polymer electrospinning and chondrocyte implantation [12–14]. Cell electrospraying, also known as bio-electrospraying, has been used to implant cells in this case [15–20]. By combining both techniques, several microintegration approaches have been developed, but contradictory results have been reported. Braghirolli *et al* developed stem cell-laden poly(lactide-co-glycolide) (PLGA) electrospun scaffolds, using a parallel polymer electrospinning-cell electrospraying strategy. Still, the electrostatic repulsion between the polymer and cell suspension jets resulted in low cell integration efficiency into the fibres [15]. On the other hand, Canbolat *et al* implemented a multi-layered approach, by alternating between polymer electrospinning and cell pipetting, resulting in high viability of the fibroblasts incorporated into the polycaprolactone (PCL) fibres [16]. Here, the electrostatic repulsion of the jets, reported by Braghirolli *et al* [15], and the consequent cell loss was prevented [16]. In this regard, this multi-layered strategy can be adapted to cartilage TE to develop tissue constructs, by alternating polymer electrospinning with chondrocyte electrospraying, allowing the automated chondrocyte incorporation during the fabrication of the scaffolds. However, there are reports that emphasize that the use of toxic solvents for polymer electrospinning, cells' exposure to the electric field, the evaporation of the culture medium, and resulting cell dehydration, and the process environmental conditions (low CO_2 concentration and low temperature) during cell electrospraying can negatively influence the chondrocyte viability [16,21–23]. To overcome these issues, the formation of a shield/protection around the chondrocytes during electrospray can be useful. Polymeric supplements with a high viscosity and low conductivity can be added to the cell suspension to protect cells from the electric field and delay the rate of dehydration during the electrospray process [24–26]. The goal of this study was to improve chondrocyte

viability in a multi-layered fabrication process by determining the viability of the electrosprayed chondrocytes embedded within PCL/Gelatin electrospun mesh, as well as the resulting mechanical properties, using various types and concentrations of polymer supplements (Gelatin and Alginate) on the chondrocyte suspension. It is hypothesized, that increasing supplement concentrations will improve the survivability of electrosprayed chondrocytes as well as the mechanical properties of the multi-layered construct. Although the depth-controlled chondrocyte distribution was not evaluated in this instance, the electrosprayed chondrocyte viability assessment performed in this study is considered a critical first step to validate the use of this multilayer process to create tissue constructs depth-controlled chondrocyte distribution.

MATERIALS AND METHODS

All experiments were performed under non-sterile conditions, using a NANON 01 electrospinning machine (MECC; Fukuoka, Japan) (Figure 1a), thoroughly cleaned with 70 % (v/v) ethanol aqueous solution (ChemLab) beforehand. The remaining used instruments were already sterile or autoclaved at 121 °C beforehand. All the electrospray and electrospinning systems were assembled and cleaned prior to use with a phosphate-buffered saline (PBS; Sigma-Aldrich) supplemented with 1% (v/v) penicillin/streptomycin (P/S; Sigma-Aldrich) and 2.5 µg/mL solution of Amphotericin B (Sigma-Aldrich).

3.1.1. Fabrication of the chondrocyte-laden electrospun meshes

3.1.2. Preparation of the polymeric solution

The polymer solution of PCL (Sigma-Aldrich; 80 kDa) and Gelatin from porcine skin (Sigma-Aldrich) was dissolved separately in 2,2,2-trifluoroethanol (TFE, TCI) at a concentration of 10 wt% and stirred vigorously at room temperature until complete dissolution. Before electrospinning, the PCL and Gelatin solutions were mixed in a 70:30 volume ratio, respectively, and a small amount of acetic acid (0.2 % (v/v), Sigma-Aldrich) was added. The resulting solution was filtered-sterilized using a 0.45 µm filter and poured into a 5 mL plastic syringe.

3.1.3. Preparation of the chondrocyte-laden electrospray solutions

The immortalized human chondrocyte cell line, C28/I2 (kindly provided by Prof. Mary Goldring, Hospital for Special Surgery, New York and Harvard University), was maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air, in Dulbecco's Modified Eagle Medium (DMEM)/Nutrient Mixture F-12 Ham (F-12) 1:1 v/v (DMEM – Gibco, Life Technologies; F-12, Sigma-Aldrich), supplemented with 10 % (v/v) non-heat inactivated fetal bovine serum (Gibco,

Life Technologies), 1 % (v/v) Penicillin/Streptomycin (Grisp) and 0.25 µg/mL Amphotericin B. The medium was refreshed twice a week. Chondrocytes were harvested at pre–confluence using trypsin/EDTA solution (0.05%/0.02%, Sigma-Aldrich) and split into six groups, each with 1.0×10^6 chondrocytes in 300 µL of culture medium: control group, where non–electrosprayed chondrocytes were maintained in the laminar flow hood at room temperature during the electrospraying process; electrosprayed group without supplement; electrosprayed groups with 1.5 and 3 wt% Gelatin; electrosprayed groups with 1.5 and 3 wt% Alginate. Gelatin and Alginate solutions were prepared by dissolving Gelatin from porcine skin and sodium alginate from algae (Sigma-Aldrich) in culture medium, respectively, and then the solutions were filter-sterilized.

3.1.4. PCL/Gelatin electrospinning and chondrocyte electrospraying with and without supplements

This protocol was carried out in a similar manner for all groups, except for the control group. All experiments were performed at room temperature (20-25 °C) within a humidity range of 30 and 40%. The PCL/Gelatin electrospinning solution and the respective chondrocyte suspension were placed in the electrospinning machine (Figure 1a). A petri dish (100 mm diameter) with a sheet of aluminium foil connected to the ground through a copper wire – collector – was placed 15 cm below the needles' orifice (Figure 1b). First, the PCL/Gelatin solution was electrospun for 5 minutes at 1.5 mL/h at 27kV through a 21G blunt needle (0.51 mm diameter and 1.5 mm length) (1^{st} layer). Then, the chondrocyte suspension was electrosprayed on top of the polymeric layer for 9 minutes at 2 mL/h at 10 kV through a 27G blunt needle (0.21 mm diameter and 1.5 mm length) at a needle to collector distance of 5 cm (2^{nd} layer, Figure 1d). A final PCL/Gelatin layer was electrospun for 5 minutes under the same conditions to seal the chondrocytes into the construct (3^{rd} layer). The final 3-layered constructs, illustrated in Figure 1c, were incubated for 2 hours at 37 °C, and then detached from the aluminium foil, cut into 15 mm squares, placed in 24-well plates (Figure 1e), and cultured for 7 days. The 3-layered constructs possessed a thickness of nearly 100 µm. The control group consisted of 1.0×10^6 chondrocytes, that were not subjected to electrospraying, statically seeded onto PCL/Gelatin meshes, previously electrospun under the referred conditions (1.5 mL/h at 27kV through a 21G blunt needle placed at a distance of 15 cm from the collector) and sterilized in 70 % (v/v) ethanol aqueous solutions, and also cultured for 7 days. The medium was refreshed two times a week. A $n = 6$ was considered for each group.

3.2. Characterization of the electrospun mesh and the supplemented chondrocyte-laden electrospray solutions

3.2.1. Fibre diameter and pore size

The morphology of single PCL/Gelatin electrospun meshes was visualized via scanning electron microscopy (SEM) (Hitachi TM4000 plus, Japan) at an accelerating voltage of 5 kV. Based on the SEM images, fibre diameter and pore size distributions were determined using Image JPro Plus software: fibres diameter was analysed manually by measuring the diameters of over 200 randomly selected fibers of each scaffold formulation ($n > 200$); pores sizes were manually measuring over 100 randomly selected areas between the fibers ($n > 100$).

3.2.2. Viscosity measurements

Dynamic viscosity of the supplemented chondrocyte-laden electrospray solutions ($n = 3$) was measured using a SNB-2-H Digital Viscometer (MTI corporation) with Spindle 1. Measurements were performed at 25ºC with 33% humidity, at a rotating speed between 6 to 60 rotations per minute.

3.2.3. Conductivity measurements

The conductivity of the supplemented chondrocyte-laden electrospray solutions ($n = 3$) was measured using a conductivity sensor from Mettler Toledo. Measurements were performed at 25ºC.

3.2.4. In vitro degradation assessment

The *in vitro* degradation analysis of the PCL/Gelatin electrospun meshes with supplemented electrosprayed suspensions was carried out by incubating the constructs, with known weights, in PBS at 37 °C for 7 days. After 1, 4, and 7 days of incubation, samples ($n = 3$) were taken from each group, washed in distilled water and freeze-dried. After weighting the samples, the percentage of polymer remaining in the scaffold was estimated, as previously described [27].

3.3. Characterization of the chondrocyte-laden electrospun meshes

3.3.1. Chondrocyte viability

Chondrocyte metabolic activity was assessed using resazurin reduction assay at day 1, 4 and 7. Briefly, a resazurin solution (0.1 mg/ml; ACROS Organics) in PBS was added to culture medium at a final concentration of 10 % (v/v). Chondrocytes were incubated in this solution at 37 °C for 4 h in the dark, after which 100 µL per well was transferred to a 96–well plate and absorbance at 570 and 600 nm was measured. For each day, final absorbance values for each sample were

calculated as the ratio Abs570/Abs600 nm minus the Abs570/Abs600 nm ratio of a negative control (PCL/Gelatin mesh immersed in culture medium). The absorbance values of the control group on the first time point were taken as 100 % and cell viability calculated as a percentage of these values.

3.3.2. Chondrocyte distribution

After 7 days of culture, the constructs were removed from culture, fixed in 4 % (w/v) paraformaldehyde (ACROS Organics) in PBS, permeabilized with 0.1% v/v Triton X–100 (Fisher Scientific), stained for nuclei (4',6–diamidino–2–phenylindole, DAPI; Sigma–Aldrich) and then visualized from a top view perspective using a fluorescence microscope (Axioimager M2, Zeiss) with magnification of 20×/0.50.

3.3.3. Topographic visualization and chondrocyte morphology

The final constructs were dehydrated with increasing concentrations of ethanol aqueous solutions (50, 70, 90, 95 and 100 % v/v), treated with hexamethyldisilane (HMDS; TCI), kept overnight in a fume hood for air drying, mounted in an aluminium stub and observed from a top view perspective using a Hitachi TM4000 plus at an accelerating voltage of 5 kV.

3.3.4. Mechanical testing

Rectangular-shaped samples of the PCL/Gelatin meshes and the final constructs after 7 days of culture were stretched, using a Shimadzu MMT–101N (Shimadzu Scientific Instruments, Japan) with a load cell of 100 N, at a constant cross–head speed of 10 mm/min (n = 4), after a preload of 0.01 N. Specimens were incubated in PBS for 24 hours before the test. The thickness of each sample was measured separately using a micrometre. The Young's moduli of the samples were calculated through the tangent modulus of the linear portion of the stress-strain curve obtained at low strain values (30%).

3.4. Statistical analysis

Data are expressed as mean ± standard deviation. Statistical significance was determined, using OriginLab, by performing as suited One-way analysis of variance (ANOVA) and Two-way ANOVA – to evaluate the effect of each of the two independent variables (supplement and concentration) and their interaction –, all followed by post hoc Tukey's test. Significance was accepted at p-values less than 0.05.

RESULTS

Uniform, smooth and beadless single PCL/Gelatin electrospun meshes were obtained (Figure 2a), with an average diameter of 0.33 ± 0.11 µm (Figure 2b) and an average pore size of 4.99 ± 1.32 µm, with the higher pore percentage within the 4 and 6 µm range (60 %; Figure 2c). The thicknesses of the 3-layered constructs were 60 ± 4.08, 93.75 ± 4.79, 117.5 ± 12.58, 90 ± 7.07 and 106.25 ± 10.31 µm for 0 wt%, 1.5 wt% and 3 wt% Alginate, 1.5 wt% and 3 wt% Gelatin, respectively. The viscosity and conductivity of the supplemented chondrocyte-laden electrospray solutions was measured, and the results are displayed in Figure 3. An increase in supplement concentration generated a corresponding increase in the electrosprayed solution viscosity, regardless of the supplement used. Nevertheless, a substantially higher increase was observed for the Alginate-based solutions (from 6.68 ± 2.67 mPa.s at 0 wt% to 99.27 ± 1.84 mPa.s at 1.5 wt% ($p < 0.05$) and 397.47 mPa.s ± 10.39 at 3 wt% ($p < 0.05$)), compared to the Gelatin-based solutions (from 6.68 ± 2.67 mPa.s at 0 wt% to 29.74 ± 2.86 mPa.s at 1.5 wt% ($p < 0.05$) and 107.10 ± 6.32 mPa.s at 3 wt% ($p < 0.05$)). Alginate-based solutions conductivity increased significantly with alginate concentration (from 14.88 ± 0.14 mS/cm at 0 wt% to 16.62 ± 0.08 at 1.5 wt% ($p < 0.05$) and 18.28 ± 0.17 at 3 wt% ($p < 0.05$)). On the other hand, the opposite tendency was observed for the Gelatin-based solutions (from 14.88 ± 0.14 mS/cm at 0 wt% to 14.62 ± 0.09 at 1.5 wt% ($p < 0.05$) and 14.14 ± 0.02 at 3 wt% ($p < 0.05$)).

In vitro degradation studies were performed to assess the influence of the incorporation of the supplements on the biodegradability of the multi-layered constructs and the results are shown in Figure 4. All constructs lost polymer content throughout the first day of incubation. (86.68 ± 1.23 % for 0 wt%, 79.63 ± 3.14 % for 1.5 wt% Gelatin, 73.79 ± 2.15 % for 3 wt% Gelatin, 78.63 ± 0.76 % for 1.5 wt% Alginate and 75.94 ± 1.55 % for 3 wt% Alginate). Both Gelatin and Alginate-supplemented constructs displayed substantial less polymer content after the 7-day period incubation, particularly the 3 wt% constructs (68.62 ± 0.79 % for Gelatin and 69.13 ± 1.13 %), while the 0 wt% construct displayed a minor weight loss in comparison (83.56 ± 1.68 %). Note that the amount of electrosprayed supplement in both cases did not exceed 4 % of the total polymer content.

To assess the effect of the multi–layered approach and the incorporation of supplements to the chondrocyte suspensions, viability assays were performed on the embedded electrosprayed chondrocytes and the results are displayed in Figure 5. A statistically significant difference was found between the control (100 ± 6.50 %) and the electrosprayed group (11.55 ± 2.67 %, $p < 0.05$) on day 1. Nevertheless, the percentage of viable electrosprayed chondrocytes increased

substantially over the 7-day culture time (from 11.55 ± 2.67 % at day 1 to 22. 25 ± 4.29 % at day 7, $p < 0.05$). A similar trend was observed for the electrosprayed chondrocytes resulting from chondrocyte suspensions containing 1.5 wt% (from 13.88 ± 5.44 % at day 1 to 22. 36 ± 9.90 % at day 7, $p < 0.05$) and 3 wt% Gelatin (from 14.83 ± 1.22 % at day 1 to 28. 41 ± 2.35 % at day 7, $p < 0.05$) and containing 1.5 wt% (from 15.24 ± 5.18 % at day 1 to 24. 77 ± 4.57 % at day 7, $p < 0.05$) and 3 wt% Alginate (from 32.87 ± 2.16 % at day 1 to 50. 89 ± 9.21 % at day 7, $p < 0.05$). To evaluate the effect of different supplements and the respective concentrations on electrosprayed chondrocyte viability, a Two-way ANOVA was performed, and the results are displayed in Table 1. Statistically significant differences were observed on the percentage of viable electrosprayed chondrocytes between Gelatin and Alginate ($p < 0.001$), as well as between 1.5 and 3 wt% ($p < 0.001$), regardless of the time point. The *p*-values obtained from the Tukey-tests results, shown in Table 2, revealed that not only 3 wt% Alginate–laden chondrocyte suspensions generated significantly higher electrosprayed chondrocyte viability ($p < 0.001$) than any other condition, but Gelatin incorporation to the chondrocyte suspension did not impact significantly the percentage of viable electrosprayed chondrocytes compared to the chondrocyte suspension alone ($p > 0.05$).

SEM and DAPI staining images of the chondrocytes (Figure 6) revealed that chondrocytes were, in fact, embedded and attached within the PCL/Gelatin fibres. Moreover, the highest number of chondrocytes visible corresponded to highest alginate concentration (3 wt%), which was consistent with the viability results. Additionally, a more uniform distribution of chondrocytes was observed when Alginate was used as a supplement.

The mechanical properties of the final multi-layer constructs were evaluated under tension and the results are shown in Figure 7. The Young's modulus of PCL/Gelatin meshes was significantly lower (0.44 ± 0.29 MPa, $p < 0.05$) than of 3 wt% constructs. Moreover, an increase of the average value of Young's Moduli was observed with an increasing concentration, regardless of the supplement used. Indeed, Young's modulus increased from 0.93 ± 0.69 MPa for 0 wt% to 4.71 ± 1.82 MPa for 3 wt% Gelatin and 7.85 ± 0.24 MPa for 3 wt% Alginate. The effect of different supplements and their concentrations on the Young's Moduli of the final constructs was determined once more using a Two-way ANOVA (Table 1), revealing that while no statistically significant differences were found between the two supplements, a substantially higher Young's Moduli was observed for the 3 wt% concentration relative to 0 wt% (without

supplement). The *p*-values from the Tukey-tests results (Table 2), on the other hand, showed no significant differences between any supplement-concentration interaction.

DISCUSSION

The purpose of this study was to see how the multi–layered combined approach as well as and the use of natural polymeric supplements – Gelatin and Alginate – at different concentrations, affected the viability of electrosprayed chondrocytes embedded in electrospun PCL/Gelatin mesh and the mechanical properties of the final multi-layer construct. The polymeric blend of PCL and Gelatin used in this work for the fibre formation has been already reported for cartilage TE [27–29]. In this case, Gelatin offered cell recognition domains (RGD) and improved wettability for superior chondrocyte attachment, as well as maintaining an optimal viscosity behaviour – avoiding capillary instabilities of the jet at the tip of the needle – and the creation of beadless and uniform nanofibers [27,28]. Additionally, the insertion of various Gelatin concentrations enables the final fibre diameter to be tuned and regulated. [27]. The electrospun PCL/Gelatin mesh process parameters were previously determined [27] and therefore, the electrospinning process occurred very smoothly. Although the small pore sizes of the electrospun meshes were insufficient to ensure cell migration through the layers, they were sufficient to assess the survivability of chondrocytes exposed to electrospraying and integration on multilayer constructions, as previously demonstrated [27,30,31].

The chondrocyte metabolic activity of the constructs was determined using the Resazurin method, which measures the conversion of resazurin (7-hydroxy-3H-phenoxazin-3-one-10-oxide), a blue non-fluorescent compound, to resorufin, a highly fluorescent and pink compound, triggered by dehydrogenase of metabolic active cells in response to changes in the reducing environment within the cytoplasmic matrix. [32]. This conversion is proportional to the number of viable cells in the constructs, and it can be used to determine chondrocyte viability. Electrosprayed chondrocyte initial viability was strikingly low – less than 15 % –, suggesting that, despite the reported potential of PCL/Gelatin blend system, chondrocyte attachment and viability was not improved. Several reports have attributed this phenomenon to cell exposure to the electric field, contact with toxic solvents and dehydration conditions [15–24]. To reduce the effect of these agents, Stankus *et al* used media supplemented with Gelatin for cell spraying, resulting in improved cell response [24]. So, it was first hypothesised that the presence of these supplements as well as an increase in their concentrations in the

chondrocyte suspension would improve the viability of the electrosprayed chondrocytes within the electrospun PCL/Gelatin mesh. However, the results reported here contradict this idea to some extent. In fact, adding Gelatin to the chondrocyte suspension had no effect on chondrocyte viability. On the contrary, Alginate generated a near 2-fold increase on chondrocyte viability at 3 wt% compared to the other conditions, for all time points, which was in agreement with the SEM and DAPI staining images. These results suggest that this particular combination of supplement and concentration was able to provide chondrocytes with a shield/protection while being electrosprayed. It should be emphasized that 3 wt% was the maximum supplement concentration used in these experiments, because higher concentrations would significantly increase the viscosity of the chondrocyte suspension, resulting not only in difficulties on the stabilisation of the electrospray jet, but also on increased shear stresses of passing through the cell electrospraying apparatus on chondrocytes [14]. A possible explanation for the difference on electrosprayed chondrocyte viability between the two supplements, particularly at 3 wt%, can be their different rheological and electrical properties. Indeed, according to the results, the Gelatin-based solutions had a significantly lower viscosity than the Alginate-based solutions, which is in agreement with previous reports [33–36]. In comparison to the Gelatin supplement, this allowed for considerably less chondrocyte dehydration (due to the resulting lower evaporation) following electrospraying, resulting in higher chondrocyte viability. In contrast to previous reports [37–41], a similar trend was observed for the conductivity of supplemented solutions, though to a smaller extent. The influence of the viscosity, however, had the major impact on chondrocyte viability.

So far, to the authors' knowledge, no studies have reported the electrospraying of chondrocytes and its combination with polymer electrospinning complicating the comparison of the present results. Nevertheless, several reports have explored this approach for direct incorporation of cells on scaffolds during electrospinning [13–20]. Indeed, Ehler and Jayasinghe developed highly cellularized 3D cardiac patches using a coaxial approach, where no distinguishable differences were found between the control cells and electrospun cells, achieving viabilities as high as 80 % [17]. Paletta *et al*, on the other hand, employed a parallel approach combining electrospinning of poly-(l-lactic acid) and electrospraying of osteoblasts, determining that osteoblast viability was dependent on several electrospinning process parameters, such as electrospraying distance and duration of the experiments, ranging from 55 to 66 % [19]. A similar strategy was followed by Braghirolli *et al*, where, despite the electrostatic repulsion

between the PLGA and cell jets, embedded stem cells still retained a 89 % viability, and were able to proliferate and colonize the scaffold [15]. To prevent this electrostatic repulsion, Stankus *et al* employed a perpendicular microintegration approach to incorporate smooth muscle cells (SMC) into poly(ester urethane)urea (PEUU) fibres, resulting in thick constructs with uniform cellular integration and cell viability greater than 90 % [18]. Canbolat *et al*, on the other hand, reported a cell layering technique, alternating between PCL electrospinning and manual cell seeding – sandwiching fibroblasts between two electrospun nanofibrous mats –, initially obtaining approximately 60 % viability, later reduced by residual solvent cytotoxicity [16]. While no direct comparisons of the obtained results can be made since different cell types, cell placement techniques, electrospraying and electrospinning parameters were used in previous reports, it can be concluded that the cell viability obtained in this study – when no supplement was used – was substantially lower (< 15 %). Nonetheless, supplementing the cell suspension with 3 wt% Alginate resulted in a 2-fold increase on this percentage, which, being still lower than those reported in previous works, it was enough to allow considerable chondrocyte proliferation in a 7-day period.

Uniaxial tensile assays were used to investigate the mechanical properties of the final multi-layer structures, as well as the effect of varying doses of Gelatin and Alginate supplements on the stiffness of the multilayered PCL/Gelatin constructs. While most studies examining the mechanical behaviour of native articular cartilage and/or engineered cartilage focus on compression, native articular cartilage is subjected to a complex loading environment that includes not only compression, but also shear and tension, whether unconfined, confined, or *in situ*. [27,42]. In fact, articular cartilage's tensile characteristics play a key role in its mechanical function, particularly in the superficial zone [43,44], that these constructs resemble. In this instance, a reduction of the final constructs' Young's Modulus upon incorporation of chondrocytes or polymeric supplementation, was expected. Indeed, Stankus *et al* reported substantially lower Young's Modulus of the SMC-microintegrated PEUU relative to the polymer alone [24]. Moreover, reduced construct mechanical properties were reported upon supplementation of the media with Gelatin, which was attributed to the disruption of the fibres network due to Gelatin gelation [24]. Also, Braghirolli *et al* observed breaks in the fibre network due to cellular placement [15]. However, in this work, this was not observed. Chondrocyte–laden constructs were stiffer than the PCL/Gelatin-only constructs, particularly when 3 wt% supplements were used. Since this multi-layered approach alternates between PCL/Gelatin

electrospinning and chondrocyte electrospraying, it is hypothesized that the fibrous network disruption described by Stankus *et al* [24] did not occur. In fact, this behaviour was observed even when no supplement was used, even though no statistically significant differences were detected, implying a Gelatin dissolution event into the electrosprayed culture medium. This phenomenon, previously reported by Semitela et al [27], might be increasing the overall construct stiffness as the amount of Gelatin fibres within the electrospun meshes could be decreasing, and it might be amplified by the supplements. Indeed, the *in vitro* degradation assay demonstrated that the highest weight loss was observed for the 3 wt% constructs lost the most weight, implying that a greater amount of polymer leached into the culture medium. Given that prior research has established that PCL did not degrade over time [27], weight loss might be due to the release of Gelatin and/or Alginate from the constructs. A similar conclusion has been reported by Xu *et al*, using a combined PCL and Pluronic F-127 electrospinning and chondrocyte gel printing [45]. Considering the Young's Modulus of the superficial zone of the articular cartilage to be within the 2-50 MPa [43,46], the Young's modulus of multilayered constructs with supplements could be included into the lower limit interval range of native superficial zone, suggesting its potential for cartilage tissue engineering.

Even though further optimization of this multi-layered approach should be performed, particularly regarding chondrocyte viability percentage and the production of hyaline-like cartilaginous matrix within the fibres extending the culture time, the promising results reported here using polymeric supplements on chondrocyte suspension for multi-layered electrospinning and electrospray fabrication processes demonstrate that these technologies could be implemented to create tissue constructs wherein depth-controlled chondrocyte distribution is incorporated into construct fabrication for the development of 3D chondrocyte–laden scaffolds for cartilage TE.

CONCLUSION

The addition of 3 wt% Alginate to the electrospray chondrocyte suspension resulted in a significant improvement in chondrocyte viability when compared to the case without polymer supplement, showing that this supplement could protect chondrocytes from the electrospraying process. The introduction of chondrocyte and polymer supplements into the PCL/Gelatin fibres increased the final constructs tensile properties. These findings further suggest that this multi-layered approach may be applied to cartilage TE, allowing for automated

chondrocyte incorporation during scaffold fabrication, and therefore a depth-controlled chondrocyte distribution.

ACKNOWLEDGEMENTS

The authors acknowledge the financial support through project POCI-01-0145-FEDER-028424-PTDC/EME-SIS/28424/2017, which was funded by the Operational Program for Competitiveness and Internationalization (COMPETE 2020) in its component FEDER and by Science and Technology Foundation (FCT) through the OE budget. The authors also thank to FCT for the PhD grant SFRH/BD/133129/2017.

DATA AVAILABITILTY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

REFERENCES

[1] L. Zhang, J. Hu, K.A. Athanasiou, The Role of Tissue Engineering in Articular Cartilage Repair and Regeneration, Crit. Rev. Biomed. Eng. 37 (2009) 1–57. doi:10.1615/CritRevBiomedEng.v37.i1-2.10.

[2] L. Han, A.J. Grodzinsky, C. Ortiz, Nanomechanics of the Cartilage Extracellular Matrix, Annu. Rev. Mater. Res. 41 (2011) 133–168. doi:10.1146/annurev-matsci-062910-100431.

[3] S. Kazemnejad, M. Khanmohammadi, N. Baheiraei, S. Arasteh, Current State of Cartilage Tissue Engineering using Nanofibrous Scaffolds and Stem Cells, Avicenna J. Med. Biotechnol. 9 (2017).

[4] I. Jun, H.-S. Han, J. Edwards, H. Jeon, Electrospun Fibrous Scaffolds for Tissue Engineering: Viewpoints on Architecture and Fabrication, Int. J. Mol. Sci. 19 (2018) 745. doi:10.3390/ijms19030745.

[5] A. Sensini, C. Gotti, J. Belcari, A. Zucchelli, M.L. Focarete, C. Gualandi, I. Todaro, A.P. Kao, G. Tozzi, L. Cristofolini, Morphologically bioinspired hierarchical nylon 6,6 electrospun assembly recreating the structure and performance of tendons and ligaments, Med. Eng. Phys. 71 (2019) 79–90. doi:10.1016/j.medengphy.2019.06.019.

[6] A.F. Girão, Â. Semitela, G. Ramalho, A. Completo, P.A.A.P. Marques, Mimicking nature:

Fabrication of 3D anisotropic electrospun polycaprolactone scaffolds for cartilage tissue engineering applications, Compos. Part B Eng. 154 (2018) 99–107. doi:10.1016/j.compositesb.2018.08.001.

[7] A. Keirouz, G. Fortunato, M. Zhang, A. Callanan, N. Radacsi, Nozzle-free electrospinning of Polyvinylpyrrolidone/Poly(glycerol sebacate) fibrous scaffolds for skin tissue engineering applications, Med. Eng. Phys. 71 (2019) 56–67. doi:10.1016/j.medengphy.2019.06.009.

[8] J. Wu, Y. Hong, Enhancing cell infiltration of electrospun fibrous scaffolds in tissue regeneration, Bioact. Mater. 1 (2016) 56–64. doi:10.1016/j.bioactmat.2016.07.001.

[9] J.M. Ameer, A.K. PR, N. Kasoju, Strategies to Tune Electrospun Scaffold Porosity for Effective Cell Response in Tissue Engineering, J. Funct. Biomater. 10 (2019) 30. doi:10.3390/jfb10030030.

[10] P. Thevenot, A. Nair, J. Dey, J. Yang, L. Tang, Method to Analyze Three-Dimensional Cell Distribution and Infiltration in Degradable Scaffolds, Tissue Eng. Part C Methods. 14 (2008) 319–331. doi:10.1089/ten.tec.2008.0221.

[11] R.I. Issa, B. Engebretson, L. Rustom, P.S. McFetridge, V.I. Sikavitsas, The Effect of Cell Seeding Density on the Cellular and Mechanical Properties of a Mechanostimulated Tissue-Engineered Tendon, Tissue Eng. Part A. 17 (2011) 1479–1487. doi:10.1089/ten.tea.2010.0484.

[12] A.K. Ekaputra, G.D. Prestwich, S.M. Cool, D.W. Hutmacher, Combining electrospun scaffolds with electrosprayed hydrogels leads to three-dimensional cellularization of hybrid constructs, Biomacromolecules. 9 (2008) 2097–2103. doi:10.1021/bm800565u.

[13] D. Poncelet, P. de Vos, N. Suter, S.N. Jayasinghe, Bio-electrospraying and cell electrospinning: Progress and opportunities for basic biology and clinical sciences, Adv. Healthc. Mater. 1 (2012) 27–34. doi:10.1002/adhm.201100001.

[14] J.A. van Aalst, C.R. Reed, L. Han, T. Andrady, M. Hromadka, S. Bernacki, K. Kolappa, J.B. Collins, E.G. Loboa, Cellular Incorporation Into Electrospun Nanofibers, Ann. Plast. Surg. 60 (2008) 577–583. doi:10.1097/SAP.0b013e318168db3e.

[15] D.I. Braghirolli, F. Zamboni, G.A.X. Acasigua, P. Pranke, Association of electrospinning with electrospraying: A strategy to produce 3D scaffolds with incorporated stem cells for use in tissue engineering, Int. J. Nanomedicine. 10 (2015) 5159–5170. doi:10.2147/IJN.S84312.

[16] M.F. Canbolat, C. Tang, S.H. Bernacki, B. Pourdeyhimi, S. Khan, Mammalian cell viability in electrospun composite nanofiber structures, Macromol. Biosci. 11 (2011) 1346–1356. doi:10.1002/mabi.201100108.

[17] E. Ehler, S.N. Jayasinghe, Cell electrospinning cardiac patches for tissue engineering the heart, Analyst. 139 (2014) 4449–4452. doi:10.1039/c4an00766b.

[18] J.J. Stankus, L. Soletti, K. Fujimoto, Y. Hong, D.A. Vorp, W.R. Wagner, Fabrication of cell microintegrated blood vessel constructs through electrohydrodynamic atomization, Biomaterials. 28 (2007) 2738–2746. doi:10.1016/j.biomaterials.2007.02.012.

[19] J.R.J. Paletta, F. Mack, H. Schenderlein, C. Theisen, J. Schmitt, J.H. Wendorff, S. Agarwal, S. Fuchs-Winkelmann, M.D. Schofer, Incorporation of osteoblasts (MG63) into 3D nanofibre matrices by simultaneous electrospinning and spraying in bone tissue engineering, Eur. Cells Mater. 21 (2011) 384–395. doi:10.22203/eCM.v021a29.

[20] L. Weidenbacher, A. Abrishamkar, M. Rottmar, A.G. Guex, K. Maniura-Weber, A.J. deMello, S.J. Ferguson, R.M. Rossi, G. Fortunato, Electrospraying of microfluidic encapsulated cells for the fabrication of cell-laden electrospun hybrid tissue constructs, Acta Biomater. 64 (2017) 137–147. doi:10.1016/j.actbio.2017.10.012.

[21] P. Hindle, A.C. Hall, L.C. Biant, Viability of chondrocytes seeded onto a collagen I/III membrane for matrix-induced autologous chondrocyte implantation, J. Orthop. Res. 32 (2014) 1495–1502. doi:10.1002/jor.22701.

[22] T. Schneider, B. Kohl, T. Sauter, K. Kratz, A. Lendlein, W. Ertel, G. Schulze-Tanzil, Influence of fiber orientation in electrospun polymer scaffolds on viability, adhesion and differentiation of articular chondrocytes, Clin. Hemorheol. Microcirc. 52 (2012) 325–336. doi:10.3233/CH-2012-1608.

[23] J. Nam, Y. Huang, S. Agarwal, J. Lannutti, Materials selection and residual solvent retention in biodegradable electrospun fibers, J. Appl. Polym. Sci. 107 (2008) 1547–1554. doi:10.1002/app.27063.

[24] J.J. Stankus, J. Guan, K. Fujimoto, W.R. Wagner, Microintegrating smooth muscle cells into a biodegradable, elastomeric fiber matrix, Biomaterials. 27 (2006) 735–744. doi:10.1016/j.biomaterials.2005.06.020.

[25] Q. Chai, Y. Jiao, X. Yu, Hydrogels for Biomedical Applications: Their Characteristics and the Mechanisms behind Them, Gels. 3 (2017) 6. doi:10.3390/gels3010006.

[26] Z. Shi, X. Gao, M.W. Ullah, S. Li, Q. Wang, G. Yang, Electroconductive natural polymer-

based hydrogels, Biomaterials. 111 (2016) 40–54. doi:10.1016/j.biomaterials.2016.09.020.

[27] Â. Semitela, A.F. Girão, C. Fernandes, G. Ramalho, I. Bdikin, A. Completo, P.A.A.P. Marques, Electrospinning of bioactive polycaprolactone-gelatin nanofibres with increased pore size for cartilage tissue engineering applications, J. Biomater. Appl. (2020). doi:10.1177/0885328220940194.

[28] R. Zheng, H. Duan, J. Xue, Y. Liu, B. Feng, S. Zhao, Y. Zhu, Y. Liu, A. He, W. Zhang, W. Liu, Y. Cao, G. Zhou, The influence of Gelatin/PCL ratio and 3-D construct shape of electrospun membranes on cartilage regeneration, Biomaterials. 35 (2014) 152–164. doi:10.1016/j.biomaterials.2013.09.082.

[29] Y. Zhang, H. Ouyang, T.L. Chwee, S. Ramakrishna, Z.M. Huang, Electrospinning of gelatin fibers and gelatin/PCL composite fibrous scaffolds, J. Biomed. Mater. Res. - Part B Appl. Biomater. 72 (2005) 156–165. doi:10.1002/jbm.b.30128.

[30] A.F. Girão, Â. Semitela, A.L. Pereira, A. Completo, P.A.A.P. Marques, Microfabrication of a biomimetic arcade-like electrospun scaffold for cartilage tissue engineering applications, J. Mater. Sci. Mater. Med. 31 (2020) 69. doi:10.1007/s10856-020-06407-4.

[31] Â. Semitela, A.F. Girão, C. Fernandes, G. Ramalho, S.C. Pinto, A. Completo, P.A.A.P. Marques, Boosting in vitro cartilage tissue engineering through the fabrication of polycaprolactone-gelatin 3D scaffolds with specific depth-dependent fiber alignments and mechanical stimulation, J. Mech. Behav. Biomed. Mater. 117 (2021) 104373. doi:10.1016/j.jmbbm.2021.104373.

[32] J. O'Brien, I. Wilson, T. Orton, F. Pognan, Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity, Eur. J. Bochemistry. 267 (2000) 5421–5426. http://www.ncbi.nlm.nih.gov/pubmed/10951200.

[33] S. Duthen, C. Rochat, D. Kleiber, F. Violleau, J. Daydé, C. Raynaud, C. Levasseur-Garcia, Physicochemical characterization and study of molar mass of industrial gelatins by AsFlFFF-UV/MALS and chemometric approach, PLoS One. 13 (2018) e0203595. doi:10.1371/journal.pone.0203595.

[34] J. Ahmed, Rheological Properties of Gelatin and Advances in Measurement, in: Adv. Food Rheol. Its Appl., Elsevier, 2017: pp. 377–404. doi:10.1016/B978-0-08-100431-9.00015-2.

[35] S. Fu, A. Thacker, D.M. Sperger, R.L. Boni, S. Velankar, E.J. Munson, L.H. Block, Rheological Evaluation of Inter-grade and Inter-batch Variability of Sodium Alginate, AAPS

PharmSciTech. 11 (2010) 1662–1674. doi:10.1208/s12249-010-9547-0.

[36] N. Devina, Y.K. Eriwati, A.S. Santosa, The purity and viscosity of sodium alginate extracted from Sargassum brown seaweed species as a basic ingredient in dental alginate impression material, J. Phys. Conf. Ser. 1073 (2018) 052012. doi:10.1088/1742-6596/1073/5/052012.

[37] Y.O. Iwaki, M.H. Escalona, J.R. Briones, A. Pawlicka, Sodium Alginate-Based Ionic Conducting Membranes, Mol. Cryst. Liq. Cryst. 554 (2012) 221–231. doi:10.1080/15421406.2012.634329.

[38] C. Marchal, M. Nadi, A.J. Tosser, C. Roussey, M.L. Gaulard, Dielectric properties of gelatine phantoms used for simulations of biological tissues between 10 and 50 MHz, Int. J. Hyperth. 5 (1989) 725–732. doi:10.3109/02656738909140497.

[39] Y. Bu, H.-X. Xu, X. Li, W. Xu, Y. Yin, H. Dai, X. Wang, Z.-J. Huang, P.-H. Xu, A conductive sodium alginate and carboxymethyl chitosan hydrogel doped with polypyrrole for peripheral nerve regeneration, RSC Adv. 8 (2018) 10806–10817. doi:10.1039/C8RA01059E.

[40] M.A. Kandadai, J.L. Raymond, G.J. Shaw, Comparison of electrical conductivities of various brain phantom gels: Developing a 'brain gel model,' Mater. Sci. Eng. C. 32 (2012) 2664–2667. doi:10.1016/j.msec.2012.07.024.

[41] H. Kakita, H. Kamishima, Some properties of alginate gels derived from algal sodium alginate, J. Appl. Phycol. 20 (2008) 543–549. doi:10.1007/s10811-008-9317-5.

[42] J.M. Patel, B.C. Wise, E.D. Bonnevie, R.L. Mauck, A Systematic Review and Guide to Mechanical Testing for Articular Cartilage Tissue Engineering, Tissue Eng. Part C Methods. 25 (2019) 593–608. doi:10.1089/ten.tec.2019.0116.

[43] A.H. Huang, M. Yeger-McKeever, A. Stein, R.L. Mauck, Tensile properties of engineered cartilage formed from chondrocyte- and MSC-laden hydrogels, Osteoarthr. Cartil. 16 (2008) 1074–1082. doi:10.1016/j.joca.2008.02.005.

[44] K.R. Gratz, V.W. Wong, A.C. Chen, L.A. Fortier, A.J. Nixon, R.L. Sah, Biomechanical assessment of tissue retrieved after in vivo cartilage defect repair: Tensile modulus of repair tissue and integration with host cartilage, J. Biomech. 39 (2006) 138–146. doi:10.1016/j.jbiomech.2004.10.016.

[45] T. Xu, K.W. Binder, M.Z. Albanna, D. Dice, W. Zhao, J.J. Yoo, A. Atala, Hybrid printing of mechanically and biologically improved constructs for cartilage tissue engineering

applications, Biofabrication. 5 (2012) 015001. doi:10.1088/1758-5082/5/1/015001.

[46] S. Ahsanizadeh, L.P. Li, Strain-rate-dependent non-linear tensile properties of the superficial zone of articular cartilage, Connect. Tissue Res. 56 (2015) 469–476. doi:10.3109/03008207.2015.1066779.

LIST OF FIGURES

by One–way ANOVA followed by post hoc Tukey's test; *$p < 0.05$, where * denotes statistical significant differences between the chondrocyte–laden PCL/Gelatin constructs using different supplements concentrations and PCL/Gelatin meshes.

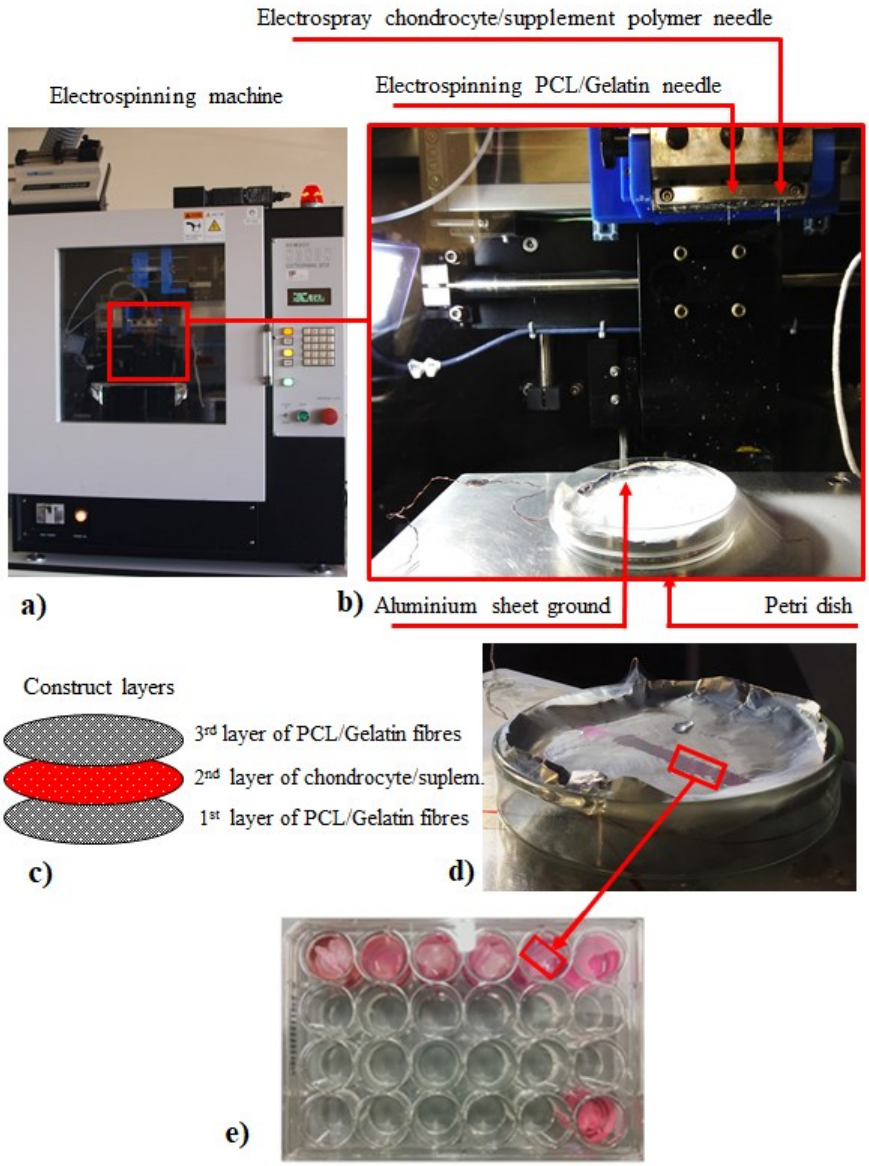

Figure 1

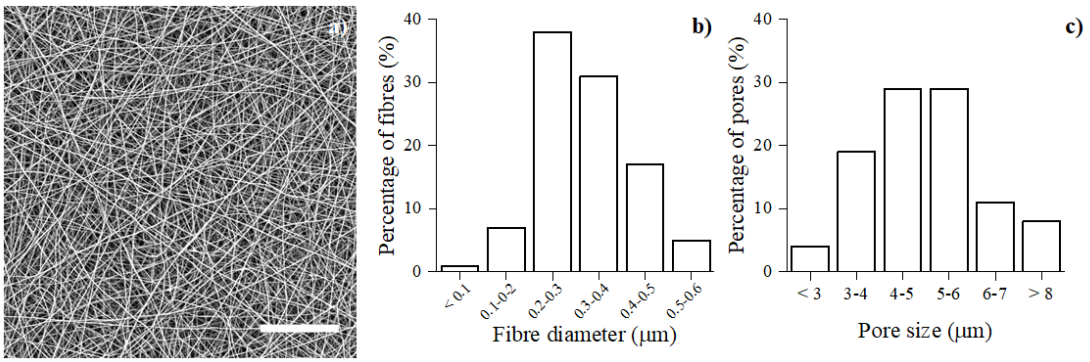

Figure 2

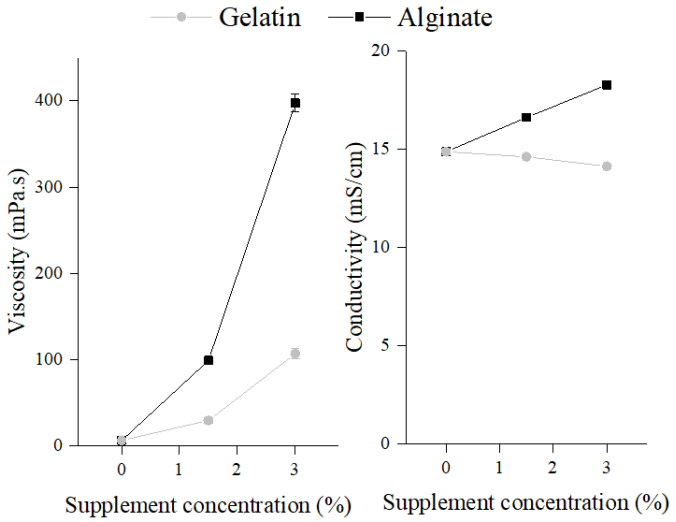

Figure 3

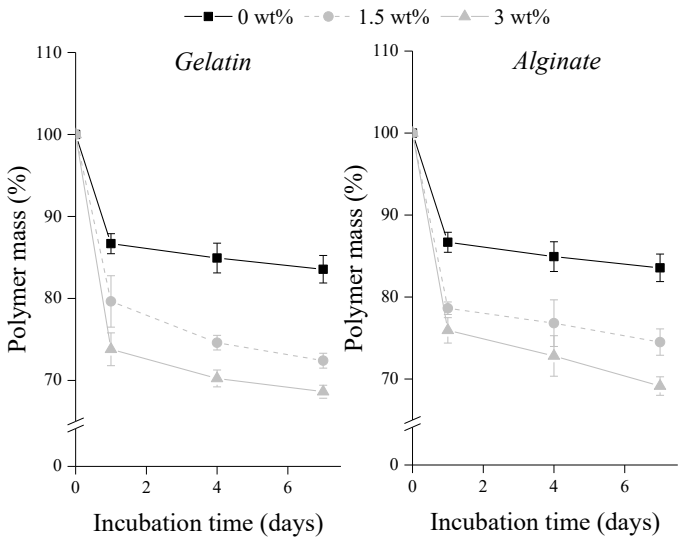

Figure 4

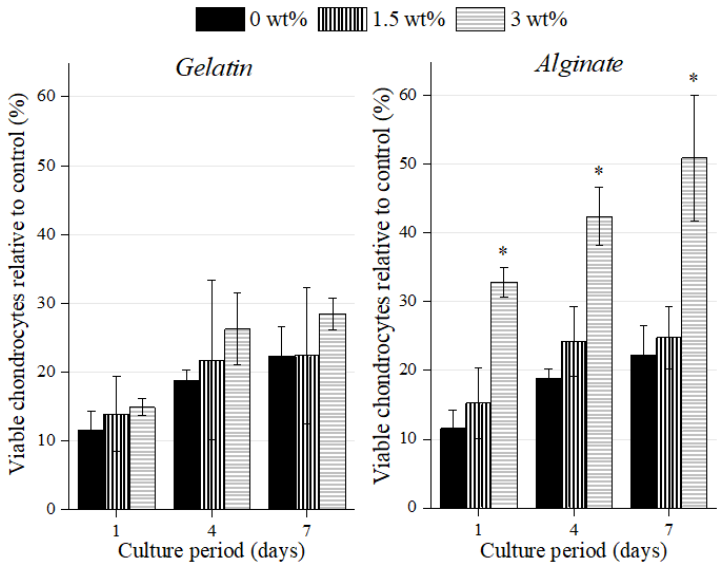

Figure 5

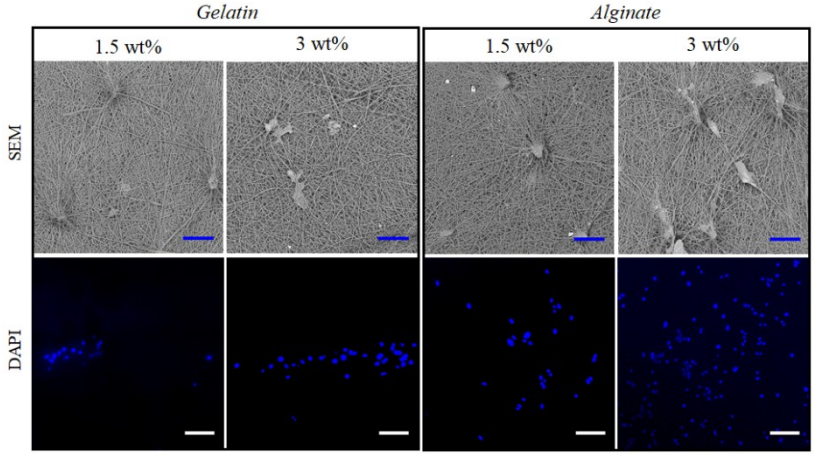

Figure 6

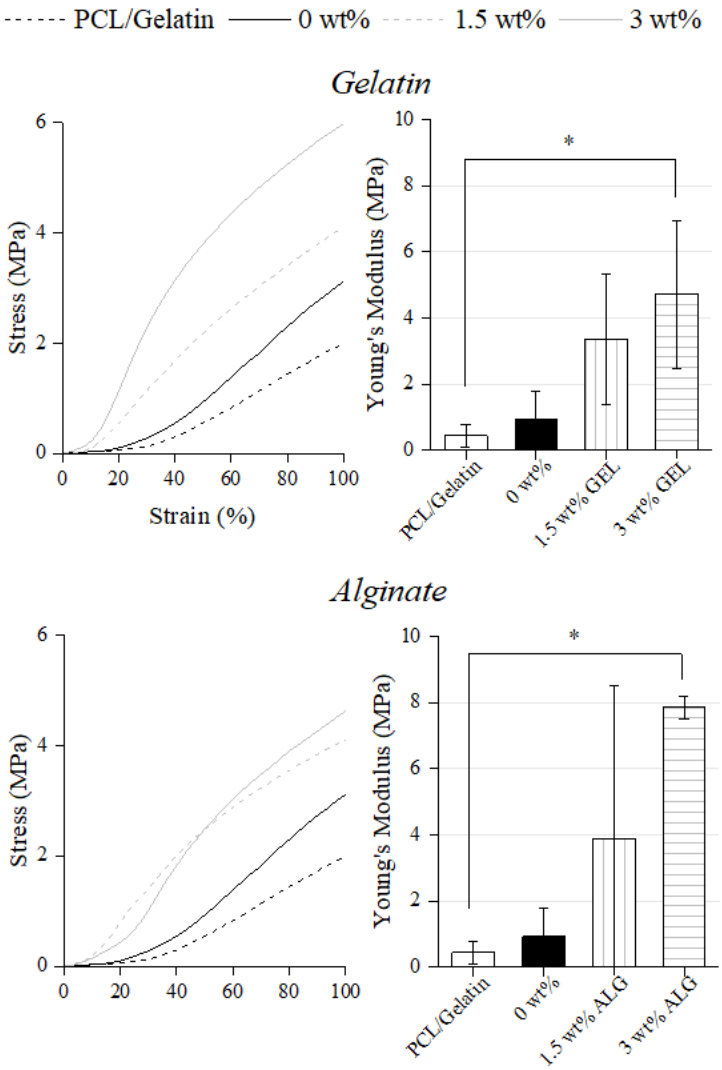
PCL/Gelatin
0 wt%
1.5 wt%
3 wt%
Gelatin
Stress (MPa)
Strain (%)
Young's Modulus (MPa)
*
PCL/Gelatin
0 wt%
1.5 wt% GEL
3 wt% GEL
Alginate
Stress (MPa)
Young's Modulus (MPa)
*
PCL/Gelatin
0 wt%
1.5 wt% ALG
3 wt% ALG

Figure 7