Title

The effect of aligned electrospun fibres and macromolecular crowding in tenocyte culture

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Abstract

Tendon injuries continuously rise, and regeneration is not only slow, but also limited due to the poor endogenous healing ability of the tendon tissue. Tissue grafts constitute the clinical gold standard treatment for severe injuries, but inherent limitations drive the field towards tissue engineering approaches to create suitable tissue constructs. Recapitulation of the native microenvironment represent a key challenge for the development of tendon tissue equivalents *in vitro* that can be further utilised as implantable devices. Methods to maintain cellular phenotype and to enhance extracellular matrix deposition for accelerated development of tissue-like modulus should be developed. Herein, we assessed the combining effect of surface topography and macromolecular crowding in human tenocyte culture. Our data demonstrated that bidirectionally aligned electrospun fibres induce physiological cell growth, whilst macromolecular crowding enhanced and accelerated tissue-specific extracellular matrix deposition. Collectively, these data advocate the use of multifactorial approaches for the accelerated development of functional tissue-like surrogates *in vitro.*

Keywords

Cell therapies; Tendon tissue engineering; *In vitro* microenvironment; Electrospinning; Macromolecular crowding; Extracellular matrix deposition

1. Introduction

Clinical data to-date demonstrate that no current therapies can restore tendon function to pre-injury state. Cell-based therapies are gaining popularity for tendon tissue engineering applications, as they utilise the cells' inherent ability to produce their own tissue-specific extracellular matrix (ECM). Tendon fibroblasts (tenocytes) and various stem cell populations are at the forefront of scientific, clinical and commercial investigation (Gaspar, Spanoudes, Holladay, Pandit, & Zeugolis, 2015; Petrou, Grognuz, Hirt-Burri, Raffoul, & Applegate, 2014). However, tenocytes readily lose their phenotype in culture (Yao, Bestwick, Bestwick, Maffulli, & Aspden, 2006) and inadequate tenogenic differentiation of stem cells is associated with ectopic bone formation (Lui, Cheuk, Lee, & Chan, 2012). Thus, recent efforts are directed towards bioinspired *in vitro* microenvironment modulators to precisely control cell fate (Spanoudes, Gaspar, Pandit, & Zeugolis, 2014). Among them, scaffolds with anisotropic structure and macromolecular crowding are under investigation as a means to maintain / induce tenogenic phenotype (Erisken, Zhang, Moffat, Levine, & Lu, 2013; Kapoor, Caporali, Kenis, & Stewart, 2010; Kishore, Bullock, Sun, Van Dyke, & Akkus, 2012; Omae, Zhao, Sun, An, & Amadio, 2009; Vermeulen et al., 2019; Yin et al., 2010) and to enhance tendon-specific ECM deposition (Gaspar, Ryan, & Zeugolis, 2019; Satyam et al., 2014). Tissue scaffolds should fulfil fundamental tissue-specific requirements for successful integration and functional outcome. These properties include cytocompatibility to encourage cell growth, infiltration and proliferation. Scaffolds should also minimise / prevent immunological response that could jeopardise the regenerative process (Beldjilali-Labro et al., 2018; Langer & Vacanti, 1993; O'Brien, 2011; Stratton, Shelke, Hoshino, Rudraiah, & Kumbar, 2016). Moreover, scaffolds should be progressively degraded by the surrounding tissue cells and body fluids, allowing cells to reproduce their natural ECM. Furthermore, any residuals of the degraded scaffold should not cause any toxic or inflammatory effects to the native cells and the surrounding tissues (Longo, Lamberti, Petrillo, Maffulli, & Denaro, 2012; O'Brien, 2011; Ramakrishna et al., 2019; Reverchon, Baldino, Cardea, & De Marco, 2012). A crucial consideration when designing implantable substrates involve the favourable mechanical properties in the range of the

specific tendon to permit an accurate replacement in the site of the lesion. Also, scaffolds need to exbibit physiological levels of stiffness and strain to permit cells proliferation and ECM synthesis (Abbah et al., 2015; J. Chen, Xu, Wang, & Zheng, 2009; Sensini & Cristofolini, 2018). Stiffness mismatch, after all, has been associated with upregulation of osteogenic markers in tenocyte cultures, even in the presence of anisotropic topography (English et al., 2015).

With regards to the structure, scaffolds should closely imitate the natural tendon formation *in vivo*. Tendons are densely packed fibrous connective tissue and exist in multiple forms and shapes in the body, but generally are composed of nanometric and hierarchically assembled collagen type I fibrils axially aligned (Spanoudes et al., 2014). Collagen alignment plays an apparent role in transmitting and absorbing the loads, the efficient structural integrity and the elongated morphology of resident cells. Out of most importance, scaffolds should exhibit suitable porosity with interconnected networks that are essential for cell infiltration, proliferation, migration, nutrient / waste transport and tissue vascularization (Causa, Netti, & Ambrosio, 2007; Chevalier, Chulia, Pouget, & Viana, 2008; Hollister, 2005).

Among the various fabrication technologies, electrospinning has been characterised as one of the most promising techniques to develop scaffolds with high levels of biomimicry for tendon tissue engineering, thanks to its ability to recapitulate the tendon tissue ECM architecture (Agarwal, Wendorff, & Greiner, 2008; Fuller, Pandit, & Zeugolis, 2014; Jiang, Carbone, Lo, & Laurencin, 2015; Liu, Thomopoulos, & Xia, 2012; Teo & Ramakrishna, 2006; Walden et al., 2017; Wang, Ding, & Li, 2013). Electrospinning is an electrically driven technology that allows to produce fibres of nanometric or micrometric diameter with varying orientations and porosity properties. The arrangement of nanofibers is based on the uniaxial stretching of a viscoelastic solution and the process requires a syringe containing the polymeric solution tipped with a metallic needle, a pump, a high voltage power source and a collector. The fibres are created when the solution is pumped at a constant flow rate and a specific voltage to develop an electric field between the needle tip and the collector. Fibre properties, such as diameter, alignment, and porosity, can be controlled by adjusting parameters, such as the type of the collector type (e.g. rotating mandrel and

parallel electrodes), solution concentration, needle gauge, extrusion rate and the distance between the needle and the collector (Braghirolli, Steffens, & Pranke, 2014; Huang, Zhang, Kotaki, & Ramakrishna, 2003; Varesano, Carletto, & Mazzuchetti, 2009).

Numerous of materials, natural and synthetic biopolymers have been employed to create aligned fibrous scaffolds that mimic tendon architecture by means of electrospinning, with variable degree of efficacy (Manea, Hristian, Leon, & Popa, 2016; Sensini & Cristofolini, 2018; Xue, Xie, Liu, & Xia, 2017). The continuous search for a 'safe' scaffolding material has intrigued us to use suture materials that have been clinically approved. Biosyn™ (Medtronic, USA), a tri-co-polymer of glycolide, dioxanone and trimethylene carbonate, holds a great potential for tendon engineering applications, as it possess favourable cytocompatibility and biodegradability characteristics, soft tissue mechanical properties and, to some extent, shape memory tendency (Thomas, Donahoe, Nyairo, Dean, & Vohra, 2011; Tomihata, Suzuki, & Ikada, 2001).

Surface topography alone is not adequate to enhance ECM deposition *in vitro*, as the *de novo* synthesised soluble procollagen is discarded during media changes. It is well documented that in the dilute conventional culture conditions, the enzymatic conversion of the water soluble procollagen to insoluble and stable collagen type I is very slow (Bateman, Cole, Pillow, & Ramshaw, 1986; Bateman & Golub, 1990; C. Chen, Loe, Blocki, Peng, & Raghunath, 2011; Hojima, Behta, Romanic, & Prockop, 1994). Macromolecular crowding (MMC) has been shown to enhance and accelerate collagen deposition in various cell populations (Benny, Badowski, Lane, & Raghunath, 2015; Benny & Raghunath, 2017; Cigognini et al., 2016; Djalali-Cuevas et al., 2019; Kumar, Satyam, Fan, Collin, et al., 2015; Kumar, Satyam, Fan, Rochev, et al., 2015; Rashid et al., 2014). Among the various crowding molecules that have been assessed to-date, carrageenan (CR), due to its inherent negative charge and polydispersity, more effectively excludes volume, inducing the highest ECM deposition (Gaspar, Fuller, & Zeugolis, 2019; Kumar, Satyam, Fan, Collin, et al., 2015; Satyam et al., 2014). Herein, we ventured to assess the synergistic effect of aligned electrospun fibres and macromolecular crowding in human tenocyte cultures.

2. Materials and Methods

2.1. Electrospinning

The production of aligned Biosyn™ fibres was achieved using the following electrospinning settings (**Table 1**) and step by step protocol:

1. A solution of 10 % w/v of Biosyn™ (Medtronic, USA) and hexafluoroisopropanol (HFIP, Sigma Aldrich, Ireland) was prepared in a 50 ml falcon tube (Sigma Aldrich, Ireland) and agitated overnight.
2. The solution was transferred to a 10 ml syringe fitted with a 27-gauge needle (Small Parts Inc.) and placed in the pump (PHD 2000, Harvard Apparatus) for electrospinning.
3. A high voltage of 22 kV was applied using a high voltage power supply (M826, Gamma High-Voltage Research, Ormond Beach, FL).
4. The electrically charged polymer ejected from the needle at a flow rate of 1.5 ml/h, was directed towards to the mandrel rotating at 3,000 rpm and placed 22 cm away from the needle.
5. The fibres were deposited onto a sheet of aluminium foil attached to the collector with a diameter of 8 cm and collected after 2 h of electrospinning.

2.2. Scanning electron microscopy (SEM)

The morphology of the fibres was assessed using SEM images (Hitachi S-2600N). Dry, unfixed samples were sputter-coated with gold (Emscope SC-500) and then imaged at 15kV. 5 distinct pieces from each sample were imaged and 3 images recorded per piece. Afterwards, the images were analysed using ImageJ software and measurements were calibrated using the scale bars within the image. Average fibre diameter was measured using 30 fibres per image.

2.3. Fibre alignment and scaffold anisotropy as judged by Fast Fourier Transform (FFT)

FFT was used to characterize fibre alignment as a function of electrospinning conditions, as previously described (Ayres et al., 2006). Briefly, 9 digitized SEM images from 3 samples were converted to 8-bit greyscale TIF files and then micrographs were processed with Image J software supported by an oval profile plug-in. A graphical depiction of the FFT frequency distribution was generated by placing a circular projection on the FFT output image and conducting a radial summation of the pixel intensities for each degree between 0 ° and 180 °, in 1 ° increments. The amount of alignment presented in the original data micrograph was reflected by the height and overall shape of the peak present in the FFT plot.

2.4. Human tenocyte isolation and culture

Tenocytes used in this study were isolated from 2 female patients (age 13 and 28) undergoing extensor digitorum tendon surgeries after obtaining all the appropriate licenses, ethical approvals, and patient consents. Cells were isolated using the migration method, as previously described (Wagenhauser et al., 2012). Briefly, tendons were cleaned aseptically with a scalpel to remove all paratendon, fat and muscle. Afterwards, tendons were cut into small pieces and placed into 6-well plate. Subsequently, tendon pieces were supplemented with 5 ml of culture media containing Dulbecco's modified Eagle medium (Sigma Aldrich, Ireland), 10 % foetal bovine serum (FBS, Sigma Aldrich, Ireland) and 1 % penicillin-streptomycin (Sigma Aldrich, Ireland) and were incubated at 37 °C in a humidified atmosphere of 5 % CO_2. Culture medium was changed every three days. After a few days, the first colonies of tenocytes were migrated around the tendon segments. When the tenocytes reached 80-90 % confluency, were treated with trypsin / ethylenediaminetetraaciticacid (EDTA) solution (Sigma Aldrich, Ireland) and sub-cultured in T-175 tissue culture flasks (SARSTEDT, Germany).

2.5. Tenocyte culture on electrospun Biosyn™ fibres

To prepare the electrospun Biosyn™ fibres for cell culture, individual pieces were cut out and placed in 24-well plates using silicone O-rings (size 111, Sigma Aldrich, Ireland). The O-rings were first sterilised by rinsing them in 70 % ethanol solution and then were washed 3 times using PBS. Electrospun fibres were then sterilised under UV for 2 h and preconditioned for 24 h using culture media. At passage three, tenocytes were seeded at 25,000 cells/cm^2 in 24-well plates and were allowed to attach for 24 h. The culture media was removed and replaced with culture media containing 100 μM L-ascorbic acid phosphate (Sigma Aldrich, Ireland) to induce collagen synthesis and with or without 50 μg/ml carrageenan (CR, Sigma Aldrich, UK). Tenocytes cultured in tissue culture plastic (TCP) without MMC treatment were taken as control. Culture media was changed every 3 days. Analysis was performed after 4, 7 and 10 days.

2.6. Cell proliferation and morphology

The cell nuclei and cytoskeleton were stained with 4',6-diamidino-2-phenylin dole (DAPI, Invitrogen, USA) and rhodamine/phalloidin (Invitrogen, USA), respectively, to quantify cell proliferation and visualise cell morphology of tenocytes cultured in electrospun Biosyn™ fibres with or without MMC. At each timepoint, the cells were fixed in 4 % paraformaldehyde (PFA, Sigma Aldrich, Ireland) for 30 min and permeabilized with 0.2 % Triton X-100 (Sigma Aldrich, Ireland) for 15 min, and stained with rhodamine / phalloidin (1:500) to mark the cytoskeleton and DAPI (1:5000) for the nucleus. Samples were imaged in an inverted fluorescence microscope (Olympus IX81; Olympus, Tokyo, Japan). The images were processed using ImageJ software. Cell proliferation was assessed by counting the number of cell nuclei using DAPI images. Particle Analyzer was used to count the number of nuclei with an area in the range of 100-1500 pixel^2, excluding the nuclei on the edges.

2.7. Cell metabolic activity assessment

alamarBlue® assay (Invitrogen, USA) was carried out to assess the effect of electrospun Biosyn™ fibres and MMC on tenocytes' metabolic activity after 4, 7 and 10 days. Briefly, at each timepoint, after removing the media, the cells were rinsed with Hanks' Balanced Salt solution (HBSS, Sigma Aldrich, Ireland) and then incubated with alamarBlue® solution (10% alamarBlue® in HBSS) at 37 °C for 4 h according to manufacturer's protocol. The absorbance was measured at 550 nm and 595 nm with a Varioskan Flash spectral scanning multimode reader (Thermo Scientific, UK). Cell metabolic activity was expressed in terms of percentage reduction of the alamarBlue® dye and normalized to the control condition of cells cultured in TCP without MMC.

2.8. Immunocytochemistry

Immunocytochemistry analysis was performed to assess the effect of electrospun Biosyn™ fibres and MMC on the arrangement and deposition of tendon-specific matrix proteins. Briefly, at each time point, tenocytes were washed with HBSS and fixed with 4 % paraformaldehyde for 30 min at ambient temperature. Cells were washed again and nonspecific sites were blocked with 3 % bovine serum albumin (Sigma Aldrich, Ireland) in PBS for 30 min. Tenocytes were then incubated overnight at 4 °C with the primary antibodies (Abcam, UK) for collagen types I (1:200, ab90935), III (1:200, ab7778), and V (1:200, ab7046) after which cells were washed 3 times with PBS, followed by 45 min of incubation at ambient temperature with the secondary antibody (Alexa Fluor 488 goat anti-rabbit, 1:400, A11034 and Alexa Fluor 488 donkey anti-mouse, 1:400 A21202; Thermo Fisher Scientific, UK). Nuclei were counterstained with DAPI. Fluorescent images were captured with an Olympus IX-81 inverted fluorescence microscope, and images were further processed with ImageJ software.

2.9. Statistical analysis

Data are expressed as mean ± standard deviation. All experiments were conducted at least in triplicates. MINITAB (version 16; Minitab, Inc.) was used for statistical analysis. Two-sample t-test for pairwise

comparisons and one-way ANOVA for multiple comparisons were performed. One-way ANOVA was employed after confirming the following assumptions: (a) the distribution of the sample mean was normal (Anderson-Darling normality test); and (b) the variances of the population of the samples were equal to one another (Bartlett's and Levene's tests for homogeneity of variance). Kruskal-Wallis test was used when either or both of the above assumptions were violated. Statistical significance was accepted at $p < 0.05$.

3. Results & Discussion

3.1. Electrospun fibre morphology, alignment and diameter

SEM images (**Figure 1A**) revealed that electrospun Biosyn™ fibres were oriented in a single direction. The fibre diameter (**Figure 1B**) ranged from 600 to 1,600 nm with the majority (~70 %) of the fibres ranging between 1,000 to 1,400 nm. The alignment of fibres was further evaluated using 2D FFT. Importantly, all analysed samples had distinctive peaks on the intensity versus degree graph, indicating that the fibres were predominately aligned in a single direction (**Figure 2**) at 90 ° degrees, as such of the collagen fibre in healthy tendons. The narrowness of the peaks corresponds to the proportion of fibres in the aligned direction, which is similar across the different samples. Most topographic strategies aim to recreate the tendon microenvironment, focusing on alignment and fibre size (Kew et al., 2011; Lin et al., 2018). It is well-documented that in soft tissues such as tendons, the smallest structural unit is the collagen molecule with a size of about 1 nm (Yang et al., 2008), which assembles into fibrils with diameters up to 360 nm (Majima et al., 2003), which then come together to form collagen fibres ranging from 1,000 to 300,000 nm, depending of the type of tendon tissue (Silver, Kato, Ohno, & Wasserman, 1992). Hence, the produced fibres are in the range of the native human tendon fibres. Additionally, tendon tissue consist of a very highly aligned collagen fibre network (Lake, Miller, Elliott, & Soslowsky, 2010), which is responsible for the exceptional mechanical properties of tendon tissues.

3.2. Cell proliferation and metabolic activity

Cell proliferation (**Figure 3A**) and metabolic activity (**Figure 3B**) were performed to evaluate the effect of aligned electrospun fibres and MMC in human tenocyte culture. Cell proliferation was increased as a function of time in culture for all groups and no statistically significant difference was observed between the groups at a given time point. Cell metabolic activity remained constant at all time points and no statistically significant difference was observed between the groups at a given time point. These results are in agreement with previous publications demonstrating that neither surface topography (English et al., 2015; Vermeulen et al., 2019) nor MMC (Cigognini et al., 2016; Gaspar, Fuller, et al., 2019; Kumar, Satyam, Fan, Collin, et al., 2015; Kumar, Satyam, Fan, Rochev, et al., 2015) negatively affect basic cellular functions.

3.3. Cell and ECM alignment and deposition

Rhodamine / phalloidin and DAPI staining were performed to analyse cytoskeleton and nuclei arrangement of human tenocytes cultured on electrospun Biosyn™ fibres, without and with MMC. Cells were randomly distributed when cultured on TCP and exhibited a pyramidal morphology with randomly oriented actin filaments. Human tenocytes maintained their physiological elongated, spindle-like morphology when cultured on the electrospun fibres, following the direction of fibre alignment at any given time point (**Figure 4**). In addition, aligned actin filaments were observed parallel to the axis of cell elongation. The addition of CR in the culture media did not affect tenocyte alignment when cultured on the fibres at any time point. In agreement with our observations, it has been demonstrated that the morphological features of the fibres can modulate cell migration, morphology and cytoskeleton organization (Baker, Handorf, Ionescu, Li, & Mauck, 2009; Bosworth, Alam, Wong, & Downes, 2013; Li, Mauck, Cooper, Yuan, & Tuan, 2007).

Immunocytochemistry analysis (**Figure 5A)** and corresponding relative fluorescence intensity analysis (**Figure 5B**) revealed minimum collagen type I deposition when tenocytes were cultured without MMC

either on TCP or on the fibres at any time point, even at day 10. Instead, from day 4, MMC significantly ($p < 0.05$) increased collagen type I deposition. Similar trend was noticed for the deposition of collagen type III, as revealed by the immunocytochemistry analysis (**Figure 6A**) and corresponding relative fluorescence intensity analysis (**Figure 6B**). Indeed, very little collagen type III deposition was observed in tenocytes without MMC treatment at any time point, whilst significantly ($p <0.05$) higher deposition was noted when MMC was used. In contrast to collagen types I and III, immunocytochemistry analysis (**Figure 7A**) and corresponding relative fluorescence intensity analysis (**Figure 7B**) for collagen type V revealed that the addition of CR in the culture media did not affect collagen type V deposition. It is worth noting that a very clear bidirectional alignment, parallel to the orientation of the electrospun fibres, of collagen types I and III was observed. The synergistic effect of surface topography and MMC on ECM matrix alignment and deposition was validated by immunocytochemistry analysis for collagen types I, III and V. These types of collagen are abundantly presented in the tendon ECM and have been characterised also, as tendon phenotypic markers *in vitro* (Gaspar, Ryan, et al., 2019; Gaspar et al., 2015; Screen, Berk, Kadler, Ramirez, & Young, 2015; Spanoudes et al., 2014; Subramanian & Schilling, 2015). A healthy tendon is mainly composed of highly organised and longitudinally oriented collagen type I fibres (Sorushanova et al., 2019; Thomopoulos, Marquez, Weinberger, Birman, & Genin, 2006). The addition of negatively charged and polydispersed macromolecules, such as carrageenan (CR), in the culture media has been shown to considerably enhance and accelerate ECM deposition in various permanently differentiated and stem cell cultures (Kumar, Satyam, Fan, Collin, et al., 2015; Lareu et al., 2007; Satyam et al., 2014), as it dramatically enhances the enzymatic processing of collagen. Collagen type III, the other main component in tendon ECM, plays a crucial role in tendon development (Buckley et al., 2013). The formation of a stable collagen type III molecule depends again, on the post-translational modification and enzymatic conversion of the *de novo* synthesised procollagen type III into the stable collagen type III (Peltonen, Halila, & Ryhanen, 1985), although the kinetics differ between collagen type I and III (Goldberg, 1977). Another important protein of tendon ECM which co-assembles with collagen

type I into heterotypic collagen fibril, contributing to collagen fibrillogenesis is collagen type V (Birk & Mayne, 1997; Sun et al., 2011). MMC did not affect collagen type V deposition, which is not surprising considering that the pericellular localization of type V procollagen has been indicated to contribute to easier access to pericellular receptors and processing enzymes, which accelerates its processing to the insoluble form (Smith, Zhang, & Birk, 2014). Of significant importance is the bidirectional orientation of the newly synthesised and deposited ECM, as this anisotropic architecture is responsible for the mechanical resilience of tendon tissues (Svensson et al., 2017).

4. Troubleshooting

4.1. Electrospinning process

1. Dripping solution from the syringe needle
    - If the polymer solution is dripping straight down from the syringe needle with no attraction to the mandrel collector, check that the conductor plate of the pump is contacting the syringe, allowing the release of the solution.
    - If the drop of polymer solution at the syringe needle is leaning towards the direction of the collector but is not forming a stream, increase the voltage. The quality of the stream can be affected by the distance of the syringe needle to the collector, therefore make sure that the distance and the voltage is adjusted until a steady stream is visible.
    - Ensure that the solution is not too dilute.
2. Globs at the syringe needle
    - If the polymer solution begins to collect and harden at the needle, turn down the voltage and swipe the glob away with a paper towel or replace the needle. Turn on the voltage and continue the electrospinning.
3. Short or discontinuous streams

- If the stream is short and discontinuous, increase the polymer solution and/or add more slow evaporating solvent and/or adjust the voltage and the distance from the needle to the collector and/or check the humidity during the electrospinning process.

4. Fibre morphology
   - If beads are found in the fibres, increase the polymer solution and ensure that the conductor plate is making continuous contact with the syringe.
   - If fibres appear as ribbons or are bleeding together, increase the concentration of the polymer or use a solvent with a higher rate of evaporation.
   - If fibres exhibit narrowest fibre diameter, increase the flow rate and/or decrease the distance from the needle to the collector and/or make sure that the humidity is less than 60 %. High humidity may decrease the average fibre diameter due to the higher evaporation of the solvent.
   - If fibre alignment is poor, increase the rotation speed.

4.2. MMC process

1. Low ECM deposition
   - Make sure that a potent crowder (e.g. polydispersed and negatively charged) is used.
   - Optimise the concentration of the crowder for the cell type under investigation.

5. Conclusions

Tissue engineering strategies focus on biomimetic approaches to encourage physiological cell growth and extracellular matrix synthesis and deposition. In tendon tissue engineering, substantial research efforts are directed towards maintaining tenocyte phenotype during *in vitro* expansion, as this particular cell population readily loses its function *in vitro*. Considering the high complexity of native tissues, single-factor approaches have failed to recreate the multifaceted *in vivo* extracellular environment, thus resulting in loss of tenocyte phenotype and function. For example, growth factor supplementation has

shown scattered results, considering the number of permutations (e.g. dose, timing, combination). Decellularised cell-derived matrices have also shown limited efficiency, as they require prolonged culture time to develop the matrix, during which the cells lose their phenotype, and the deposited matrix is of random orientation. Although decellularised tissues have shown promise, considering their limited availability, they cannot be used for both cell extraction and substrates for cell expansion. It is therefore imperative to develop bioinspired methods to control cell fate during *in vitro* cell expansion.

Electrospinning, due to its versatility, can produce scaffolds with tailored architectural features and mechanical properties to suit a wide range of clinical indications. Although in tendon tissue engineering, electrospinning is a well-established scaffolds fabrication technology, the resultants scaffolds lack a crucial element of tendon microenvironment: native tendon extracellular matrix. Macromolecular crowding is the new frontiers in tissue engineered constructs as dramatically enhances and accelerates tissue-specific extracellular matrix deposition, albeit in a random fashion. Herein, we demonstrated that the combination of anisotropic electrospun fibres and macromolecular crowding resulted in accelerated development of a more physiological tendon-like construct with bidirectionally orientated cell and extracellular matrix.

For the production of more physiological tissue-like surrogates, numerous tissue-specific modulators should be finetuned. In tendon tissue engineering, for example, mechanical stimulation and physiological low oxygen tension should be incorporated into the process to more effectively recreate *in vitro* the tendon niche. Considering that previous studies have suggested that the chemistry of the crowder affects cell phenotype, the optimal crowder for tendon cells should be identified. Previous studies have suggested that for the transition from two-dimensional to three-dimensional electrospun based constructs, higher porosity is required to allow cell infiltration. To this end, electrospinning setups that that allow / enhance cell infiltration (e.g. electrospinning with sacrificial, water-soluble, fibres) should be adopted. Considering the significant advancements of the field in the recent years, we feel that within the next decade a bioinspired tissue engineered tendon equivalent will become clinically available.

6. Acknowledgements

The authors received financial support for this work from Science Foundation Ireland, Career Development Award (Grant Agreement Number: 15/CDA/3629); Science Foundation Ireland / European Regional Development Fund (Grant Agreement Number: 13/RC/2073); H2020, Marie Skłodowska-Curie Actions, Innovative Training Networks 2015 Tendon Therapy Train project (Grant Agreement Number: 676338).

7. Conflict of Interest

The authors declare no conflict of interest. Yves Bayon is an employee of Sofradim Production, a Medtronic Company.

8. Table and figure legends

Table 1: Electrospinning parameters utilised in this study for the production of anisotropic scaffolds.

Apparatus parameters	**Electrospinning settings**
Collector	Rotating mandrel collector; diameter 8 cm
Rotation of the collector	3,000 rpm
Voltage	22 kV
Flow rate	1.5 ml/h
Distance from the ejecting needle to the collector	22 cm
Humidity	~ 50 %
Time of electrospinning	2 h

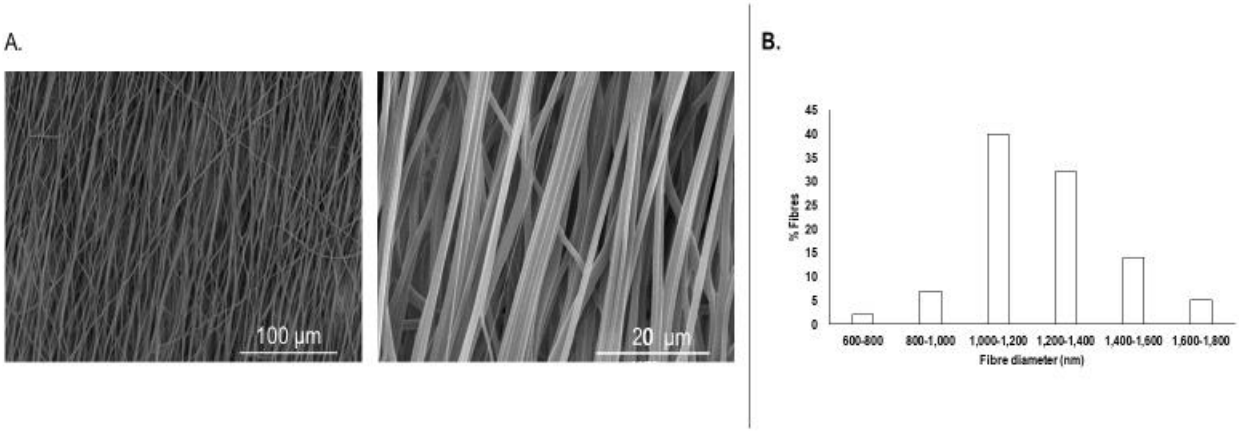

Figure 1: SEM images at 600X and 900X magnification (A) revealed that Biosyn™ fibres were aligned to a single direction. Fibre diameter analysis (B) revealed that most fibres range from 1,000 to 1,400 nm.

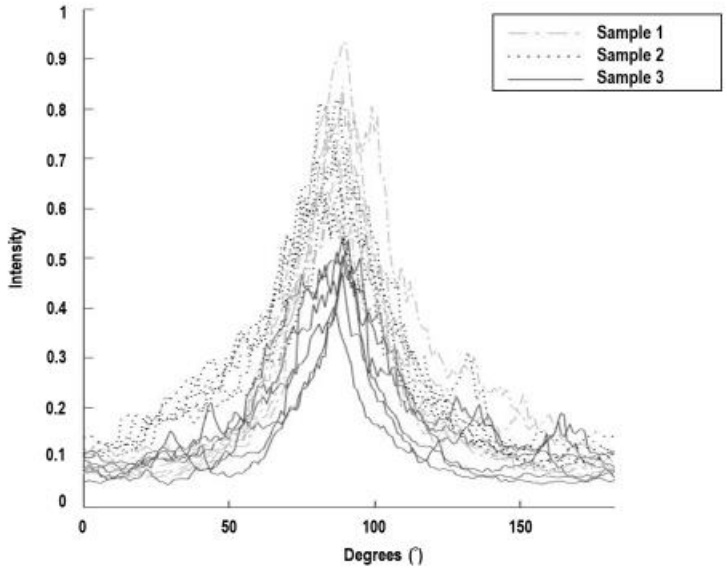

Figure 2: 2D FFT intensity vs degree graph revealed that electrospun Biosyn™ fibres were aligned to a single direction. Sample 1, 2 and 3 corresponds to the 2D FFT image analysis of individual fibres.

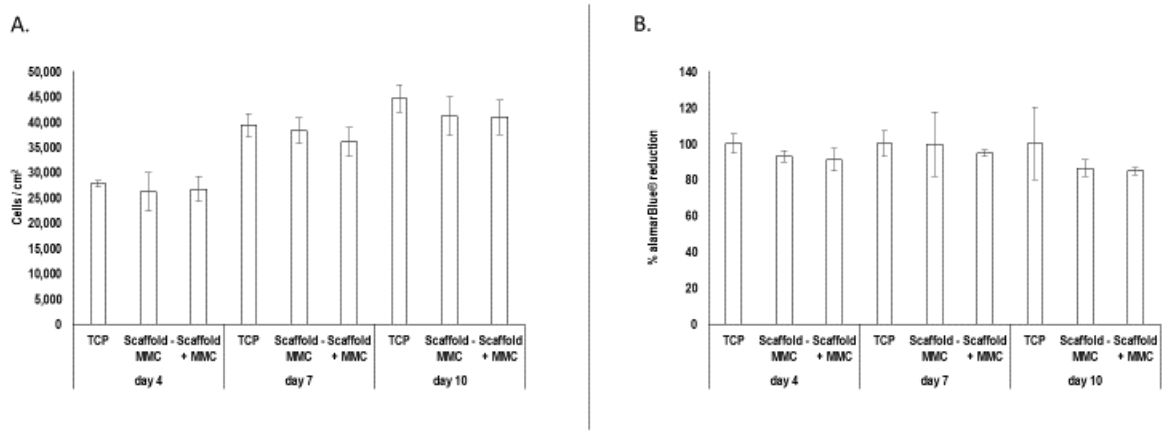

Figure 3: Cell nuclei counting (A) and alamarBlue® assay (B) revealed that cell proliferation and metabolic activity were not affected as a function of surface topography and MMC at days 4, 7 and 10.

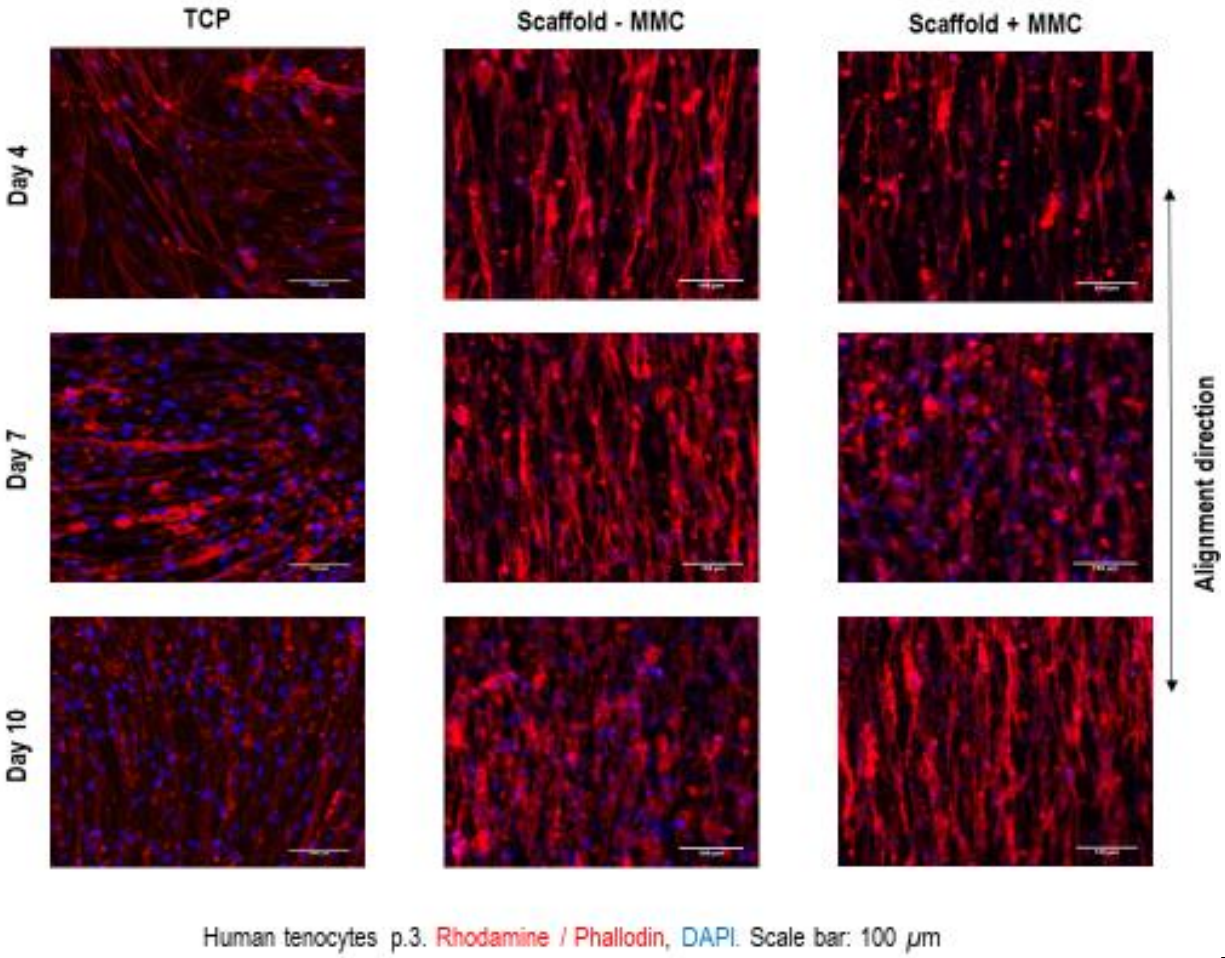

Figure 4: Cytoskeleton (rhodamine / phalloidin) and nuclei (DAPI) staining revealed that human tenocytes were aligned to the direction of the fibres after day 4, 7 and 10 when seeded on Biosyn™ scaffolds independently of the addition of CR. Cells cultured on TCP were randomly oriented.

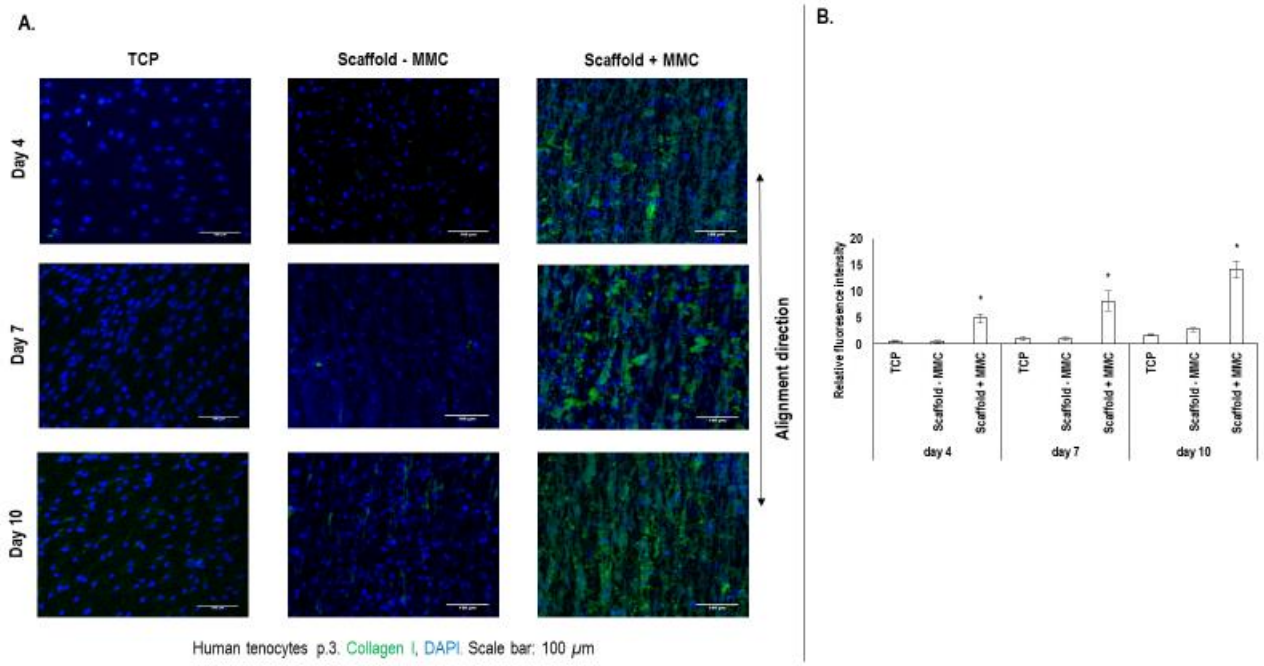

Figure 5: Immunocytochemistry analysis (A) and complementary relative fluorescence intensity analysis (B) of collagen type I revealed that at all time points, the highest ($p < 0.05$) collagen type I deposition was observed for tenocytes that were cultured under MMC conditions. Collagen type I was also aligned to the direction of the electrospun Biosyn™ fibres. * indicates statistically significant difference. Collagen type I: Green, DAPI: Blue. Scale bars: 100 μm.

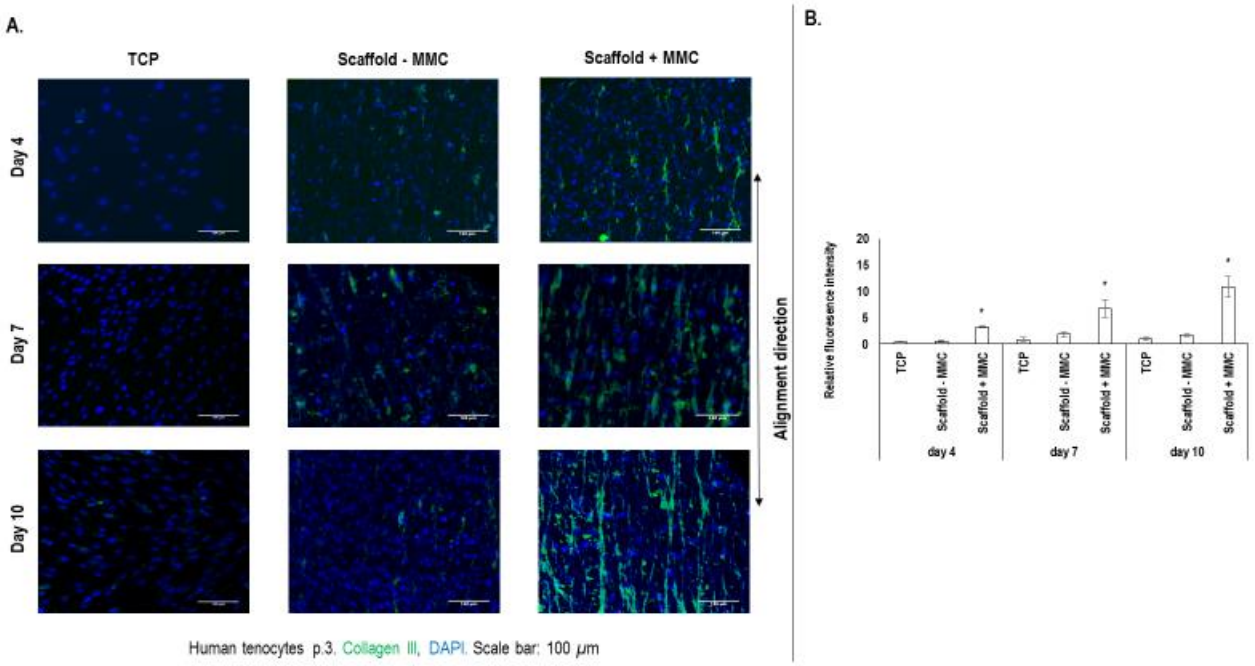

Figure 6: Immunocytochemistry analysis (A) and complementary relative fluorescence intensity analysis (B) of collagen type III revealed that at all time points, the highest ($p < 0.05$) collagen type III deposition was detected for tenocytes that were cultured under MMC conditions. Collagen type III was also aligned to the direction of the electrospun Biosyn™ fibres. * indicates statistically significant difference. Collagen type III: Green, DAPI: Blue. Scale bars: 100 μm.

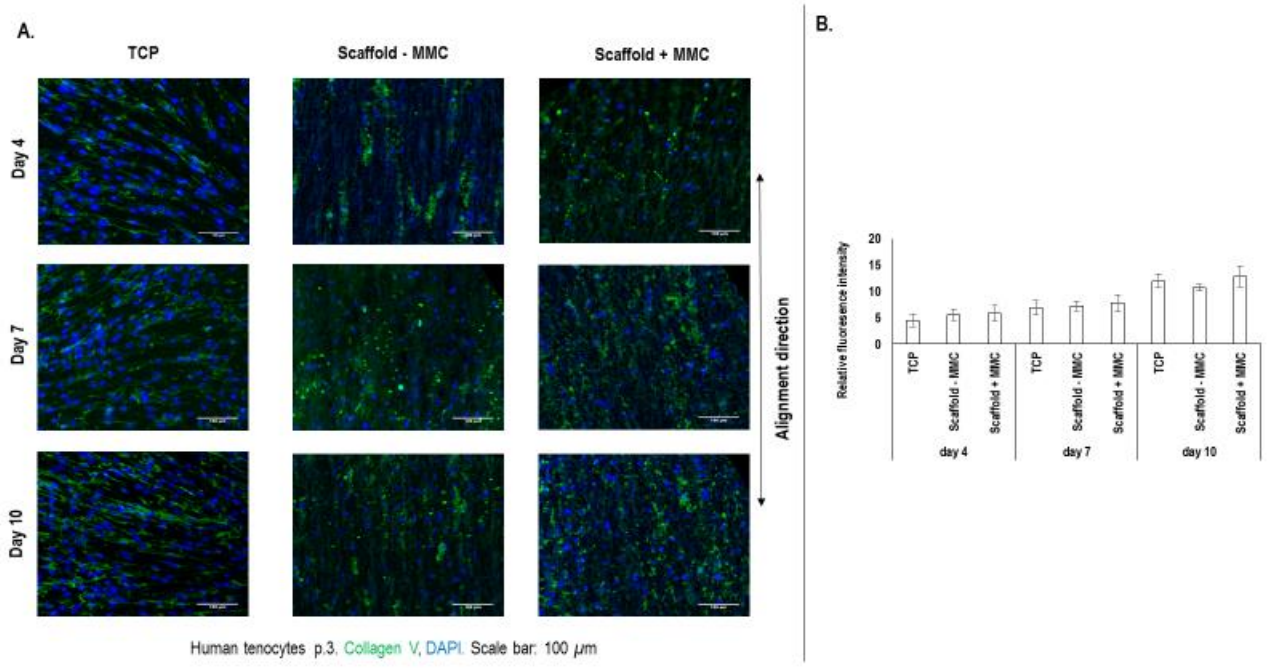

Figure 7: Immunocytochemistry (A) and complementary relative fluorescence intensity analysis (B) of collagen type V revealed that at all time points no statistical differences were observed between the crowded and non-crowded groups. Collagen type V was aligned to the direction of the electrospun Biosyn™ fibres when tenocytes were grown on them. Collagen type V: Green, DAPI: Blue. Scale bars: 100 μm.

9. References

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