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Electrospun Chitosan Nanofibers used for Chondrocyte Development: Influence of the Structure of Fibrillar Support

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Abstract

This paper describes the development of our work on electrospinning of chitosan (CS) to get pure chitosan nanofiber mats proposed as support for chondrocyte culture. Electrospinning is carried out in presence of PEO as aid, and dissolution of CS and PEO in formic acid/water mixture. The fibers were recovered using different structured collectors to evidence their role on the mat structure and advantage for cell development. Rotary cylinder, at different rotation rates, allows to align the fibers and increases their crystallinity and anisotropic mechanical performances. Square-patterned collector gives a more porous material with more cells attached. The cells fixed on nanofibers have their normal round-type morphology, contrary to the elongated structure on films or culture dish. This indicates a preservation of their original phenotype. At end, dispersion of short fibers with adhered cells is proposed for injection to repair cartilage avoiding chirurgical implantation.

Keywords

Chitosan, Chondrocytes, Electrospinning, Structured collectors, Physical properties

Introduction

Tissue engineering represents a potential approach to improve cartilage mending, where an artificial 3D extracellular matrix (ECM) is essential to generate new tissues. No current procedures for cartilage renovation have successfully achieved long-lasting regeneration and, the tissue shows little tendency for self-repair.

Native ECMs can be effectively mimicked by electrospun nanofiber membranes, specially using natural sourced polymers. In previous work, chitosan (CS)-based systems (CS and electrostatic complex CS/ hyaluronan HA) were transformed, by electrospinning, into biocompatible and biodegradable nanofibrous mats adapted for chondrocyte development [1-4]. CS materials are claimed to favor cell adhesion and growth, providing the adequate microenvironment for chondrocyte phenotype preservation. Addition of polyethylene oxide (PEO) is recognized to improve the spinnability. The PEO content in the electrospun blends is set at 20 or 30 % w/w and electrospun CS/PEO and CS/HA/PEO fibers are obtained, with diameters ranging between 100-200 nm using different solvents (acetic acid/water and formic acid/water) [5-7]. Cell proliferation tests, performed on CS fiber mats, revealed that fiber mats lead to higher proliferation rates compared to casted films used as reference [3, 4].

In this paper, our previous work is developed to study chondrocyte cell culture in dependence of the experimental conditions (structure of collectors). Several collector types allowing the production of structured nanofibrous mats are characterized before cell development.

Materials and Methods

Materials

Chitosan (CS) sample (Batch TM4778, code 42010) from Northern cold-water shrimp, *Pandalus borealis,* was obtained from Primex Ehf (Siglufjordur, Iceland), with a molecular weight (M_W) around 160 kg/mol and a degree of acetylation (DA) of 0.05, determined using ^{1}H NMR. Formic acid (FA) (ACS reagent >98%) was from Sigma-Aldrich (Product of Finland, lot #STBJ3705) and Dulbecco's Phosphate Buffered Saline (DPBS) (ref. 14190-094, Lot 2118924) from Gibco (Made in UK). Poly (ethylene oxide) (PEO) with a molecular weight of $1x10^3$ and $5x10^3$ kg/mol were used in a ratio CS/PEO = 70/30 to prepare the fibrous mat. Deionized water was used as solvent for the solutions. All reagents and polymers were used as received without further purification.

For cell culture, the C-20/A4 human chondrocyte cell line [8] was selected and the samples were seeded in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% v/v of fetal bovine serum (FBS) and a 1% v/v, in proportion with the total volume, penicillin/streptomycin/glutamine solution. Phosphate Buffered Saline (PBS) solution with a pH = 7.4, measured in the laboratory, DMEM serum-free and 0.05% Trypsin-EDTA solution were also utilized in cell experiments. All biological reagents were purchased from Gibco Life Technologies (Paisley, UK).

Electrospinning & nanofibers stabilization

Using a homemade vertical set up of the electrospinning technique, the prepared solutions were placed in a 5 mL plastic syringe fitted with a 21-gauge stainless steel needle. Then, the ensemble syringe/needle was disposed in a syringe pump (model: KDS Legato 200, KD Scientific, Holliston, MA, USA), which delivers solutions at specified flow rate. Electrospinning was effectuated applying a voltage around 25 kV between the electrodes using a dual high voltage power supplier (±20 kV, iseq GmbH, Radeberg, Germany).

The nanofibers were recovered on two different collectors: square patterned metallic collector and rotatory cylinder as shown in figure 1. The experiments were performed placing the collector at 17 cm from the tip of the needle and the operating flow rates varied from 0.02 to 0.15 mL/h. Production of fibers was carried out at room temperature in closed Plexiglas® box with relative humidity ranging between 30% and 50%. The produced nanofibers matrices were left in ambient conditions to evaporate excess of FA and water and reserved for further analyses.

X-ray Diffraction

Nanofiber mats of CS/PEO oriented fibers were analyzed on X-ray diffraction as a function of as a function of collector rotational speed. The mat was folded several times along the major orientation direction and taped on a sample holder with a pinhole. It was X-rayed with a Ni-filtered Cu Kα radiation (λ =1.542 Å), using a Philips PW3830 generator operating at 30 kV and 20 mA. Diffraction patterns were recorded on Fujifilm imaging plates placed at about 5 cm from the sample and read with a Fujifilm BAS-1800II bioimaging analyzer with 50 µm resolution.

Scanning Electron Microscopy (SEM)

SEM analyses of the samples were performed at CMTC-INP platform (Grenoble, France). The morphology of electrospun nanofiber membranes samples were observed with a scanning electron microscope Zeiss ultra 55 SEM FEG (Oberkochen, Germany), equipped with a field emission gun and operating at 1 kV. The nanofiber samples were coated with 3-4 nm gold/palladium prior to SEM imaging. The average fiber diameter (AFD) was calculated by randomly selected diameter of 500 nanofibers from each sample.

Tensile tests

The measurements were carried out using an ARES-G2 rheometer (TA Instruments, New Castle, DE, USA) equipped with grips dedicated to tensile tests. Samples were cut in rectangular shapes (6 mm x 40 mm) from the nanofibrous electrospun matrices (randomly oriented and aligned fibers), in order to maintain a free length/width ratio around 2.69. The results are expressed as the Stress σ (Pa) = Force applied (N)/section area (m^2). Tensile tests were performed starting from a zero-applied force until the material presented a breaking point, with a grip separation rate of 0.01 mm/s. The experiments were carried out at constant temperature around 25 °C. The thickness of the samples was measured with a micrometer (Mitutoyo Digimatic micrometer; up to 25 mm with precision of 0.001 mm).

Cell culture and chondrocyte development

Chondrocyte C-20/A4 initial sample was disposed in a culture flask with 20 mL of complete DMEM. Cell sample was preserved into a cell incubator (inCu safe, Panasonic) at 37 °C and 5% CO_2 constant inlet flow during few days until confluence. The nanofiber mats, having a regular shape with a surface of ~1 cm^2, were directly placed in a Petri dish and washed 2 times with the PBS solution to be subsequently hydrated in the DMEM culture solution during 2 days. After the DMEM solution being removed from the culture dish, 10 µL of a cell suspension at a concentration of $1x10^6$ cell/mL, was disposed on the fiber mat followed by the addition of 2 mL of complete DMEM. The samples were maintained into a cell at 37 °C and 5% CO_2 constant inlet flow during few days before cell growth quantification, the culture solution was renewed every 2 days. Cells were detached from the fibrous substrate and resuspended in DMEM in order to quantify the number of cells as a function of time. For this purpose, the supports were disposed in a 15 mL tube and carefully washed twice with 1 mL of PBS solution in order to remove remaining DMEM and unattached cells. Washing was followed by a detachment step consisting in the addition of 0.5 mL of Tryp-

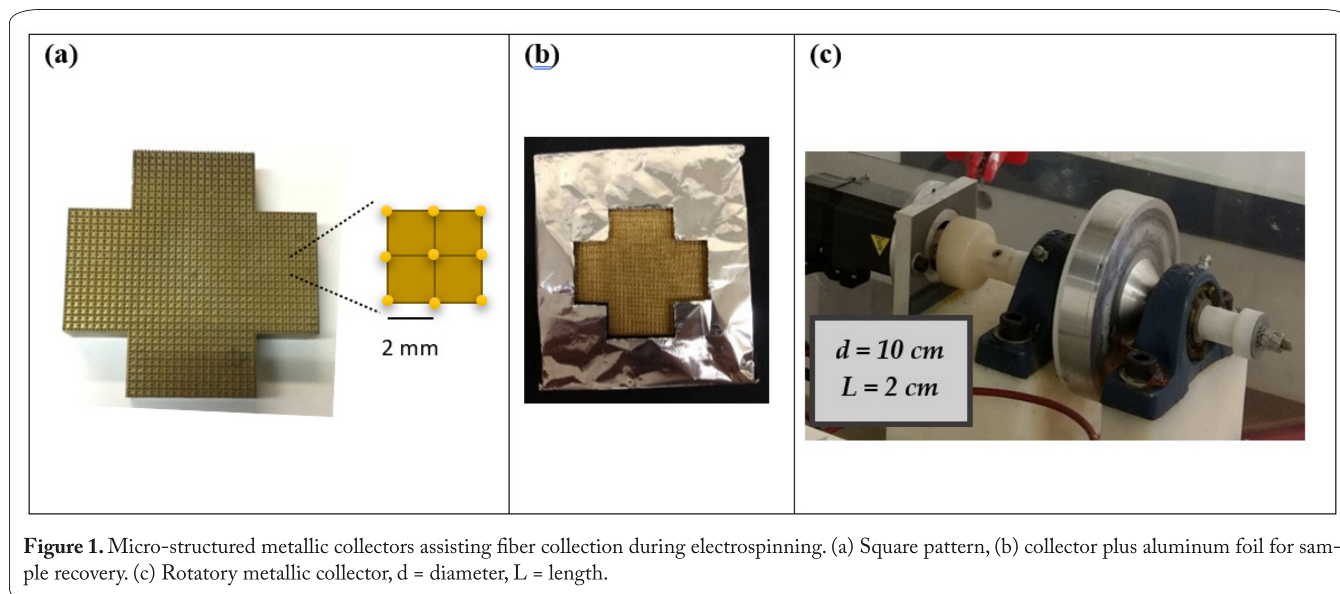

Figure 1. Micro-structured metallic collectors assisting fiber collection during electrospinning. (a) Square pattern, (b) collector plus aluminum foil for sample recovery. (c) Rotatory metallic collector, d = diameter, L = length.

sin-EDTA 0.05% and vortex agitation at 1000 rpm during 60 seconds. Further addition of DMEM and PBS washings helped to re-suspend the detached cells. Cell counting was carried out at times between 1 and 21 days after seeding and proliferation.

The final cell suspension was stained with Acridine Orange/Propidium Iodine fluorescent marker (F230001, Logos biosystems, Villeneuve d'Ascq, France) and cell quantification, in cell/mL, was performed on a dual brightfield and fluorescence cell counter (LUNA-FL, Logos biosystems, Villeneuve d'Ascq, France). This technique allows to identify and quantify the amount of total and living cells to calculate the cell viability. It gives also information about average cell size.

Cell staining

In order to qualitatively verify cell viability and visualize the presence of cells on CS-based substrates, fluorescence staining was applied. Cells were marked with Hoechst-33342, which enters in the cell nucleus and emits a blue fluorescence.

For cell visualization, the samples were kept in the well plate and were observed on an inverted fluorescence microscope Nikon Eclipse TS100. A filter was used conforming to the wavelengths of excitation and emission of the fluorochrome: *Excitation*: 325-375 nm/*Emission*: 435-485 nm. Images were acquired using the software NIS-Elements (Nikon Instruments).

Cell counting by colorimetry

For cell quantification, based on intracellular Iodonitrotetrazolium chloride (INT) reduction, the substrates were disposed in a microtube and washed with 500 µL of PBS. PBS rinsing was followed by a 10-second centrifugation cycle at 10000 g. Since cell detachment from the substrates is not needed, 500 µL of fresh DMEM and 200 µL of the INT solution were added to the sample pellet, composed of cells and the substrate, to be incubated during 1 hour at 37 °C. During incubation, INT is reduced to formazan which is visually identified by its purple-red characteristic color. Then, the culture medium is removed and the substrates are washed twice with PBS, followed by centrifugation cycles. For formazan extraction, 500 µL of DMSO are added. The microtubes are vortexed until complete dissolution of the extract. Samples were placed on a 96-well microplate by duplicate and absorbance was measured on a UV/Vis microplate spectrophotometer (Thermo Scientific Multiskan Sky Spectrophotometry) at a wave length of 490 nm. Calibration measurements were performed in order to relate a known number of cells to an absorbance value.

Fiber suspension obtention

Fibrous samples were obtained by electrospinning, set on a vertical arrangement, of the blend 70/30 CS/PEO. The process was adapted in order to reduce fiber-fiber contact during fiber collection. With the help of a basic EtOH/Water bath as fiber collector, the fiber production process was carried out. The recipient collector is placed on a metallic plate, connected to the needle tip of the polymer container (syringe) and electrospinning is performed. Pure ethanol is constantly added to compensate evaporation.

The collected fibers formed a white mat at the air/liquid interface of the bath, then, they adopted a form similar to small cotton balls as fibers were completely immerged in the basic ethanolic bath. The samples were maintained in basic EtOH/Water 80/20 v/v up to three days after electrospinning in order to assure chitosan neutralization and mat stability before further conditioning steps. The stabilized fibers were then fragmented by liquid nitrogen freezing and fiber dispersion with an Ultra-Turrax® disperser. The selection of both methods was optimized and neutralized fiber fragments were suspended in PBS at pH = 7.4.

These suspensions were centrifuged and the pellets were dried at air conditions. The concentration of fiber in the dispersion is determined by dried weight measurements, using the equation:

$$C_f\left(mg/mL\right)=\frac{W_d\left(mg\right)}{V_i\left(mL\right)}$$

Where C_f represents fiber concentration, W_d is the measured dried weight and V_i corresponds to the initial suspension volume.

For cell culture, the saline solution was extracted from the suspension by centrifugation, and replaced by DMEM added to a final fiber suspension ~ 6 mg/mL prior to cell seeding. This value was set after the initial tests were carried-out, considering that cell observation under microscope was unable in more concentrated fiber dispersions. For chondrocyte culture, 500 μL of the fiber suspension per well, were placed in 12-well plates. For cell seeding, 10 μL of a cell suspension, containing 10^6 cells per mL, were disposed and actively mixed by pipetting with the fiber suspension. Finally, DMEM was added to complete 3 mL per well and incubated for further analysis.

Results and Discussion

Influence of collector structure

Electrospinning of the systems, using micro-structured collectors allowed the obtention of randomly oriented or aligned fibers, as well as square-patterned fibrous mats. To compare fiber deposition on selected collectors, square patterned collector and rotary cylinder were utilized for electrospinning of the blend CS/PEO. The process was carried out under the conditions already described [1, 5, 6] and the mats produced are shown in figure 2.

Electrospun chitosan fibers: physical properties

Fiber morphology of the CS/PEO mats were analyzed by SEM. Samples of as-spun fiber mats were observed and average fiber diameters (AFD) were calculated by statistical size distribution. Globally, it was found that smooth fibers and homogenous substrates were obtained.

Average fiber diameters, in the range 130-170 nm, were found for the mats from both collectors: square-patterned collector and rotatory cylinder. For fibers recovered on the structured collector, it was found an AFD of 133 ± 26 nm. Similarly, the aligned fibers, using the rotating collector, presented an AFD of 167 ± 21 nm. Fiber morphology obtained by SEM is presented in figure 3.

Then, X-Ray diffraction was employed to investigate the molecular orientation and degree of crystallinity of fibers recovered on rotatory collector as a function of rate of rotation. Reflection patterns are shown in figure 4.

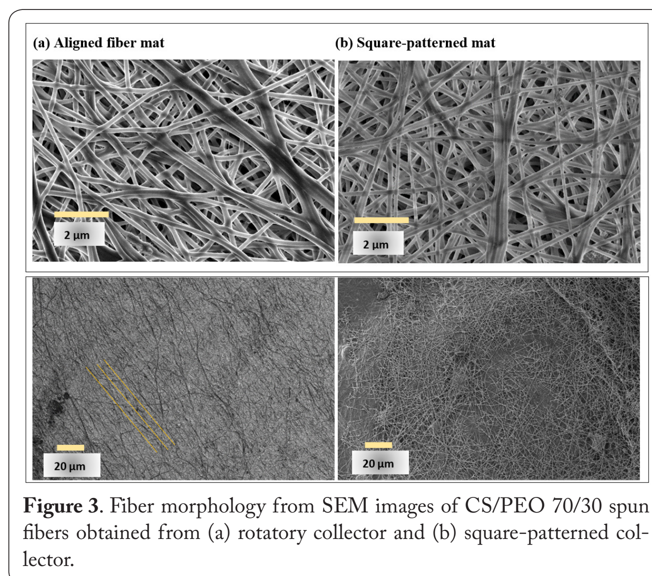

Figure 3. Fiber morphology from SEM images of CS/PEO 70/30 spun fibers obtained from (a) rotatory collector and (b) square-patterned collector.

For the CS/PEO system, during rotatory collection of fibers, their alignment was observed under microscope. At low speed (700 and 1000 rpm) fibers resemble more to a random arrangement, on the contrary at 1500 rpm, they were clearly aligned. It has been established in literature that gradual fiber alignment increases with the rotational velocity [9, 10], then a maximal orientation is achieved. It is indicated that the degree

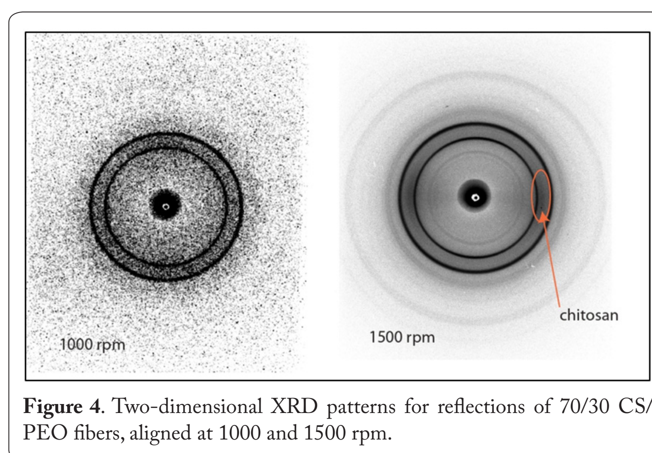

Figure 4. Two-dimensional XRD patterns for reflections of 70/30 CS/PEO fibers, aligned at 1000 and 1500 rpm.

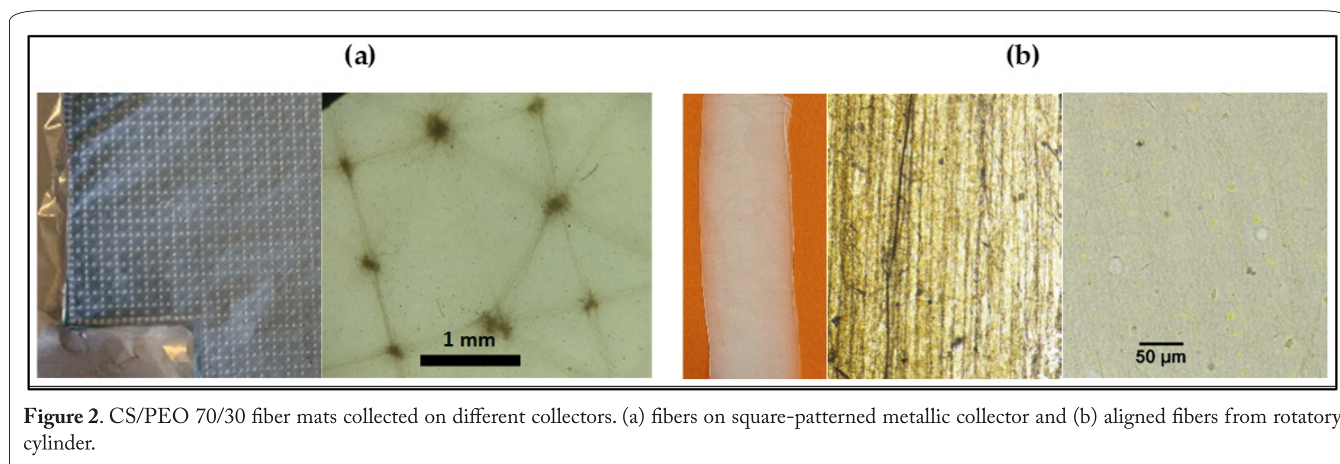

Figure 2. CS/PEO 70/30 fiber mats collected on different collectors. (a) fibers on square-patterned metallic collector and (b) aligned fibers from rotatory cylinder.

of crystallinity in the aligned fibers at 1500 rpm was higher than that of their counterparts at 1000 rpm. Therefore, crystals might be oriented parallel to the fiber axis in the aligned CS/PEO fibers at 1500 rpm as shown by XR diffraction (Figure 4).

The stress under uniaxial tension is studied on the different samples prepared (Figure 5).

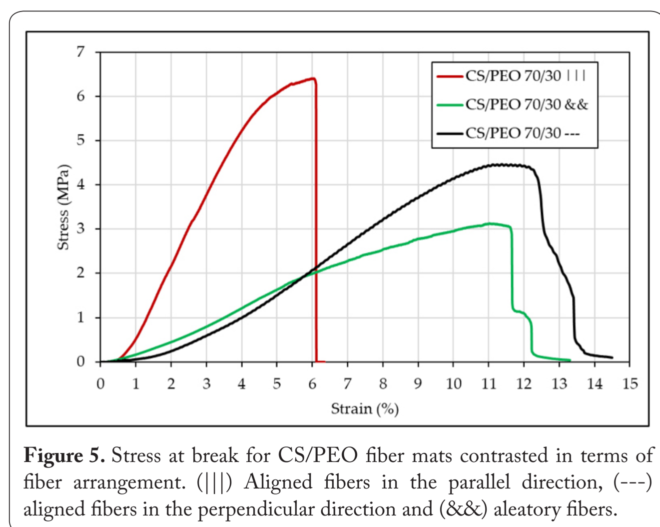

Figure 5. Stress at break for CS/PEO fiber mats contrasted in terms of fiber arrangement. (|||) Aligned fibers in the parallel direction, (---) aligned fibers in the perpendicular direction and (&&) aleatory fibers.

Orientation of CS/PEO fibers obtained with the rotating collector allows to get an anisotropic material with different mechanical behaviors along the direction of fibers and perpendicular to them. It is clear that in the direction of CS fibers, the stress at break is higher while the strain at break is significantly lower which contrasts with that randomly orientated CS fibers (ratio = 6.5/3 MPa). The mechanical behavior in the perpendicular direction is quite similar to that of random deposition. This could be in part due to connection between fibers occurring during their collection on the rotating cylinder before complete drying.

Chondrocyte development

Chondrocytes are able to attach to a substrate with a high density of cell-substrate bonds related to an adhesion strength [3]. On chitosan films, with a large available contact surface, such adhesion force leads to cell morphology changes. Porous fiber mats are observed to affect differently the cell shape. Comparison between cells adhering on fibers and on a flat surface is shown in figure 6.

From this figure, it is shown that the fibrous structure prevents changes in cell shape, cells attach all over the mat and remain spherical as found in literature [11]. This is claimed to preserve the native phenotype by keeping the same cell morphology as in original tissues [12-14].

Cell development was studied on fibers produced with the different collectors. Few results are given in figure 7.

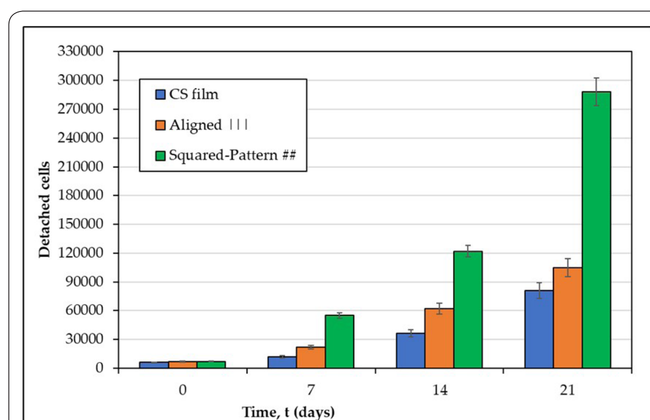

Figure 7. Cell proliferation for chondrocytes on chitosan substrates. Total living cells detached as a function of time on fibers from square-patterned collector (■), aligned fibers (■) and CS films (■). PEO, MW = 1000 kg/mol, CS/PEO 70/30. Error bars represent mean ± SD; n=4 for fiber mats and n= 3 for CS films respectively.

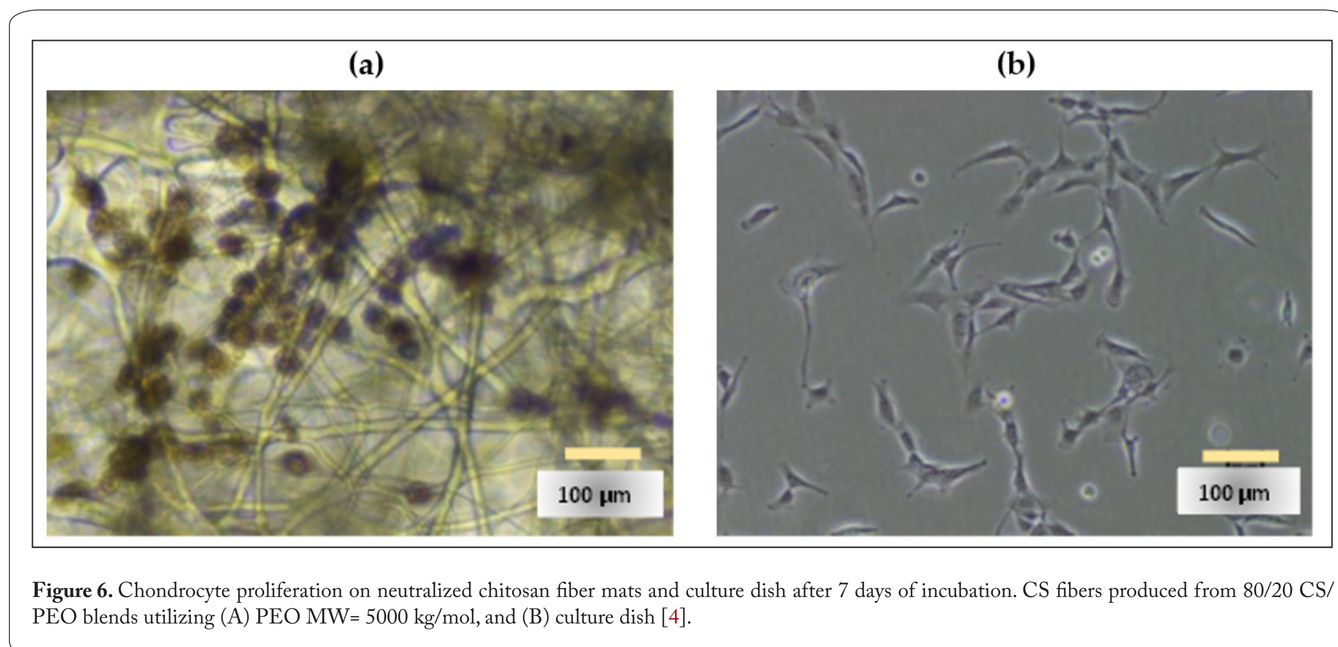

Figure 6. Chondrocyte proliferation on neutralized chitosan fiber mats and culture dish after 7 days of incubation. CS fibers produced from 80/20 CS/PEO blends utilizing (A) PEO MW= 5000 kg/mol, and (B) culture dish [4].

In the case of fibrous mats recovered on the structured collectors with squared mesh, a significant increase in detached cells quantity is found in contrast with aligned fibers and chitosan film. This substrate obtained on squared pattern collector shows lower material density which suggests they are more porous, a key property of materials for biological applications. Consequently, fluorescence microscopy observations were performed in order to reveal the cell morphology adopted once chondrocytes developed on CS fibers. Several types of substrates were utilized for cell culture as it has been presented in proliferation measurements. Development of cells on substrates obtained on the different collectors is examined after fluorescent labelling (Figure 8).

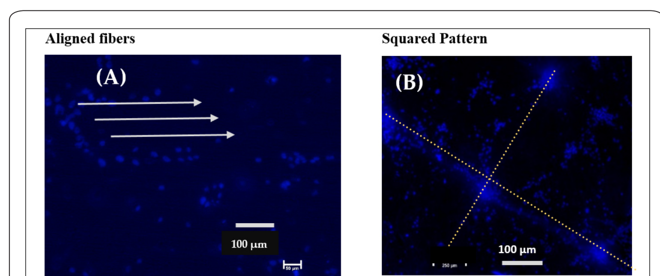

Figure 8. Chondrocytes C20A4 adhered to CS fibrous mats, observed on an inverted fluorescence microscope, 10 days after seeding. Substrates: aligned fibers (A), squared pattern (B). Magnification: 10X. Lasers: Blue, Excitation 325-375 nm/ Emission 435-485 nm.

From figure 8, it is observed that cell alignment has been partially influenced, in certain zones of the substrate, by fiber orientation. Assembly of chondrocytes, with length between 100-500 µm, are found on the mat following the same directions as fibers (figure 8A). A higher concentration of cells is mainly detected on the vertices of the patterned mat (figure 8B). This corresponds as well to a higher concentration of fibers, being the guiding points of the pattern during electrospinning, a similar conclusion was reached studying with C2C7 myoblast cultured on PLA-based structured scaffolds [15].

Compared to aligned and random fibers, cell cluster is also observed on structured collectors. Microscopy indicates an occupation cell/substrate of ~14% after 10 days of culture.

Cell adhesion and proliferation studies helped to validate the application of CS fibers as substrate towards biological approaches for cartilage regeneration with primary chondrocytes. In order to validate the biological approach, an analysis of phenotype modifications is needed in a following study. During this research, chondrocytes cultured on CS fibrous substrates were observed to maintain their native shape as shown in figure 6, which may lead to original functions preservation contrary to monolayer cultures [16].

Chondrocyte incubation on fragmented CS fibers

Even if nanomaterials based on chitosan are biodegradable in the body, a surgery is generally required to place the cell/substrate ensemble in a determined living tissue.

Then, an original development is proposed to prepare a cell/substrate suspension, able to be injected in the damaged cartilage zone. For fiber fragmentation, two techniques were applied were applied consecutively: liquid nitrogen freezing and grinding followed by fiber dispersion with an Ultra-Turrax® disperser. Colorimetry, by intracellular INT reduction, helped to improve cell counting measurements obtained by fluorescence. The assays were performed on cells adhered to fragmented CS fibers, produced from the blend CS/PEO 70/30.

In figure 9, the proliferation profile of chondrocytes C20A4 on fragmented fibers is presented and compared to proliferation rates in CS fiber mats, between 1-15 days of incubation after seeding.

From the curves shown in figure 9, cell development is confirmed by increasing measured absorbance values, in the fiber suspension, as a function of time. Cell quantities related to optical density reveals that cells could reach proliferation ratios of ~12 comparing cell seeding (t = 0) with day 15. After two weeks of incubation, After two weeks of incubation, in contrast to fiber mats, only ~75% of cells were found on fiber fragments. Even though proliferation profiles are close, the number of cells on fragmented fiber is slightly lower than on fiber mats, for a similar incubation time.

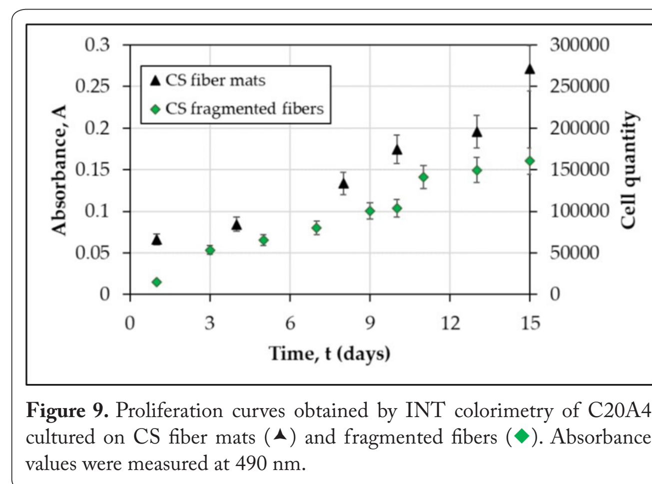

Figure 9. Proliferation curves obtained by INT colorimetry of C20A4 cultured on CS fiber mats (▲) and fragmented fibers (◆). Absorbance values were measured at 490 nm.

On this approach, it is founded possible injection of the cell/substrate ensemble for therapeutic applications. For this purpose, an experimental test based on fiber and cell injection viability was carried out. Fiber suspensions were loaded into a 5-mL syringe adapted with a cylindrical needle. Then, a flow rate of 0.017 mL/s was imposed using a KSD legato 200 infusion syringe pump. The suspensions were evaluated to pass through different needle diameters (between gauges 22-25) similar to those used for visco-supplement injections [17]. It is also observed that fragmented fibers with adhered cells interact with the native cartilage surface after being incubated during 3 days. In conclusion, the approach represents an advantageous way to place into injured articulations the necessary agents for tissue regeneration.

Conclusions

In this paper, electrospinning of chitosan is performed and applied to chondrocyte development having as objec-

tive a model for cartilage repair. In a first step, the structure of the collector was studied. For an equal sample composition, aligned fibers, from rotatory collectors, shown higher resistance to tensile traction (with anisotropy in the two senses of measurement and induction of some crystallinity) than randomly deposited fibers and structured (square mesh) fiber mats. These tests allowed to detect the more appropriate composition for the blend not only for the final application but to facilitate sample handling. Our data are also associated to the cell morphology modification when attached to film (flat) compared to pseudo spheric cell on fibers. This indicates that the chondrocyte phenotype may be preserved allowing biological activity in tissue engineering. Overall, accordingly to the obtained results, it is clearly demonstrated that pure chitosan is a good support for chondrocyte development. The porous mat should allow cell migration, preservation of cell morphology involving phenotype maintain, nutriment transport and permeability.

For cartilage repair, avoiding invasive surgeries, it was proposed to fragment fibers and prepare suspension of short fibers as support for chondrocyte development. The cell-short fibers are shown to be adapted for injection directly in damaged joints with same needle as used for HA visco-supplement injection.

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