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Original Research Article

The effect of freezing on PCL and PCL/ CS electrospun scaffolds for tissue engineering applications

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Abstract: Tissue Engineering and bio fabrication techniques play a crucial role for biomedical application solutions. The combination of biomaterials of different origin can lead to the creation of micro- and nano- structures with extra-ordinary characteristics. Currently, there is limited knowledge on how freezing protocols during cryopreservation influence the scaffolds' characteristics. In this study, we compared the influence of cryopreservation on two different scaffolds, either consisting of polycaprolactone (PCL) or PCL and chitosan (CS), fabricated by electrospinning. Their morphological and mechanical properties were evaluated, before and after cryopreservation. In our previous studies we have shown that, PCL/CS fibers were thinner, more porous, exhibiting superior mechanical properties, compared to PCL fibers. Here we concluded that the freezing process did not induce significant changes in the fibers' mechanical properties while the average diameter was slightly decreased for both types of scaffolds. In a nutshell, PCL/CS fibers can be further examined as scaffolds for cell seeding and bio preservation applications.
Keywords: Cryopreservation, Chitosan, Electrospinning, Induced nucleation, Polycaprolactone, Tissue Engineering

INTRODUCTION:

Regenerative medicine is a broad field for repair, replacement or regeneration of cells, tissues and organs that gained considerable attention during the last decades. This field embroils the application of different principles of biology and engineering to develop biological, artificial or bio-hybrid substitutes for lost or damaged tissues [1]. To support cellular migration and growth, structures such as gels, fibers and three dimensional composites are needed [2]. Electrospinning is a widely established method to fabricate micro- and nano-structured scaffolds with different shapes and sizes[3, 4].The microarchitecture of these scaffolds is similar to the extracellular matrix (ECM), while they are often used as a scaffold material in tissue engineering applications [5].Single and multi-layered fibrous scaffolds with tailor-made architecture have been previously created [6].In addition, the combination of different polymers enables the production of scaffolds with enhanced structural, physicochemical and mechanical properties [7]. Furthermore, the combination of polycaprolactone (PCL) and chitosan (CS) has been extensively used in the past with promising results [8-11].

Long term storage and high viability of cells after thawing is essential for successful approaches in tissue engineering[12].Recent developments promoted induced ice formation (nucleation) as a new factor for enhancement of the survival of cells[13].There are many ways to induce the nucleation, but the most common way is to use the cold spot nucleation[12-14].However it is not known how the induced nucleation affects the properties of electrospun materials such as non-woven fibrous scaffolds. In this study, two different scaffolds types, made by PCL or by PCL/CS, were fabricated using the blend electrospinning method as previously described [15]. We investigated the scaffolds' morphological, structural and mechanical properties before and after freezing, in order to gain better knowledge on the influence of the cryopreservation process on the scaffolds properties.

Materials and Methods:

PCL (Mn 70000-90000) and CS (medium MW) were purchased from Sigma-Aldrich. 2, 2, 2-trifluoroethanol (TFE) was purchased from abcr GmbH &Co.Kg. All materials were used as received.

Solution characteristics and preparation have been previously described [15].Briefly, a solution of PCL in TFE (190mg/ml) and a blend solution of PCL/CS in TFE (190mg/ml + 10mg/ml) were prepared and left under continuous stirring conditions for 24h in order to obtain homogenous solutions. Electrospinning was conducted under room temperature inside an electrical field of 1.15kV/cm. The solution flow rate was 1ml/h and the typical spinning time for one sample was 1h. Afterwards, the specimens that were deposited on the collector's surface were removed and kept under vacuum for 24h for the remaining solvent to evaporate.

For the evaluation of the freezing effect on fibers, they were placed into 1.8mLcryovials (Nalgene, Thermo Fisher scientific, Germany) and 2mL of 4°C cold phosphate buffered saline (PBS, Biochrom, Germany) was added. To mimic the cryopreservation of engineered tissues, scaffolds were equilibrated at 4°C for 10min and cryopreserved similar as previously described [12]. Briefly, cryovials were placed into the nucleation device, in a controlled rate freezer (CM2000, Carburos Metalicos, Spain) and cryopreserved using the following freezing program. Cryovials were cooled from 4°C with a cooling rate of 7.5°C/min down to the nucleation temperature of -6°C. After all samples achieved the desired nucleation temperature, the ice formation was induced by a cold spot nucleation. The temperature was monitored by T-type thermo element placed next to the cryovials. Nucleation was detected as a temperature rise resulting from the latent heat of fusion of water. The previous cooling rate was resumed to7.5°C/min to -30°C followed by 3°C/min until -80°C. After reaching -80°C, the cryovials were transferred into an electric freezer at -150°C (MDF-1155, Sanyo GmbH, Germany) for further analysis for different periods of time (1dand 7d). After the indicated time period the cryovials were transferred from the electrical freezer to a 37°C warm water bath and gently rocked until only a small ice crystal remained. Scaffolds were removed and placed on a filter paper for at least 24h to dry before further analysis.

100mm^2 square strips were carefully punched out from PCL and PCL/CS scaffolds and were coated with AU/Pd for 30s. Pictures of the specimens before and after freezing were obtained using a scanning electron microscopy instrument (S3400N, Hitachi) under high vacuum and high voltage (15kV), at different magnifications. The analysis of the average fiber diameter and the scaffold morphology were performed using ImageJ (National Institutes of Health, USA). Cyclic tensile tests were performed using a uniaxial tensile instrument (LM1 Electro force, TA Instruments), equipped with a 200N load cell. Rectangular, 15x10 mm strips were punched out of the electrospun specimens and were tested at 0-30% strain, 1Hz, at room temperature (n=5). The applied force and the local principal strain were monitored, and Young's modulus was calculated.

Statistical analysis was performed using one-way ANOVA with post-hoc Tukey via the software Origin Pro 8.5 (Origin Lab Corporation).

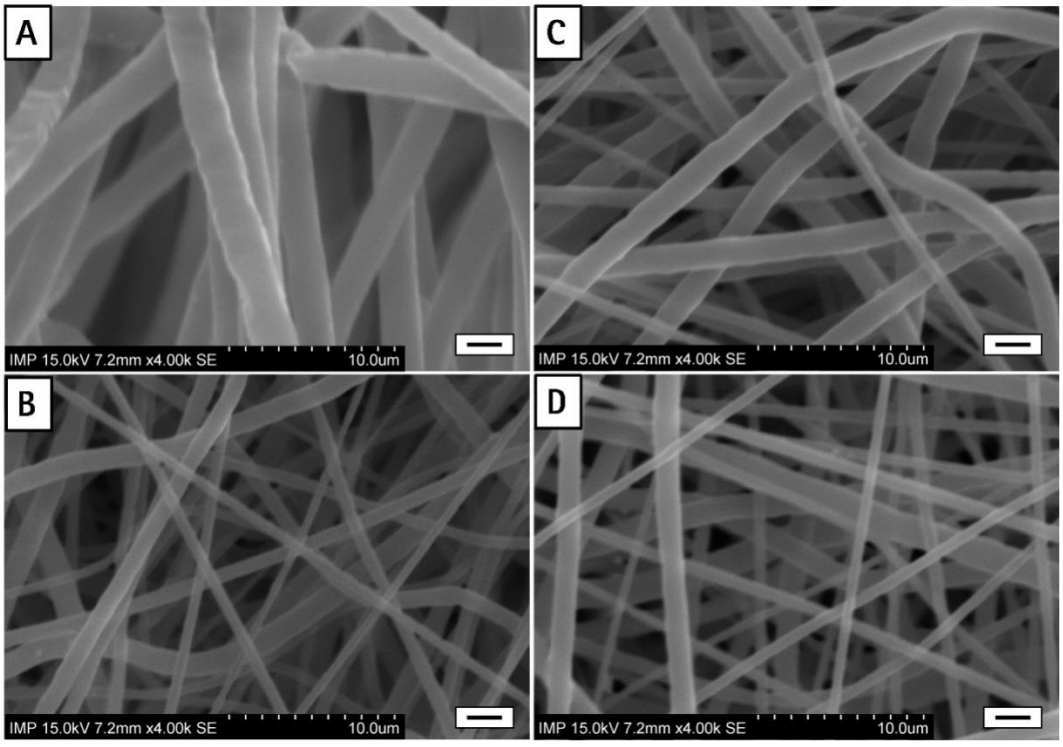

Fig 1: SEM pictures of electrospun fiber mats made by PCL (A) and PCL/CS (B), before cryopreservation; SEM pictures of electrospun fiber mats made by PCL (C) and PCL/CS (D), after cryopreservation; magnification=4000x, scale bars=2µm, voltage=15kV, electron beam to samples' surface distance=7.2mm.

RESULTS AND DISCUSSION:

The obtained SEM images highlighted that the electrospun PCL and PCL/CS fibers had a smooth surface and cylindrical shape with no apparent orientation and homogenous distribution on the surface of the collector (Figures 1A and 1B). The morphology of the fibers was not influenced by the freezing process and the structural characteristics remained similar as before cryopreservation (Figures 1C and 1D). Moreover, the average fiber diameter of the PCL and PCL/CS scaffolds before and after cryopreservation was calculated (Figure 2). PCL fibers had an average diameter of 1.77±0.30μm and PCL/CS fibers had an average diameter of 1.09±0.43μ, being significantly thinner ($p<0.001$). The fiber diameter was found not to be significantly different after cryopreservation for both types of scaffolds with the average values of 1.73±0.37μm and 1.02±0.42μm, for PCL and PCL/CS fibers respectively ($p>0.05$). In addition, the average fiber diameter of the PCL scaffolds remained significantly higher than the one of the PCL/CS scaffolds ($p<0.001$).

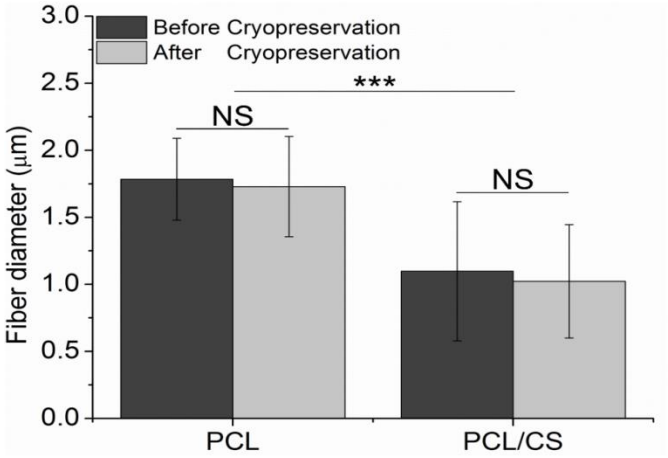

Fig 2: Influence of cryo preservation on the average fiber diameter of PCL and PCL/CS scaffolds; n=50, mean±SD, (*=p<0.001, NS=p>0.05).**

The average Young's modulus values before freezing for PCL and PCL/CS scaffolds were 43.27±6.51MPa and 49.36±3.68MPa, respectively (Figure 3) as previously reported [15]. After one day in the freezer, the average values for PCL and PCL/CS scaffolds were 44.94±11.31MPa and 43.71±8.32MPa, respectively. In addition, after seven days in the freezer, the average values for PCL and PCL/CS scaffolds were 50.73±9.18MPa and 49.8±5.6MPa, respectively (Figure 3). From the obtained data, it can be concluded that the average Young's modulus values were not significantly affected by the freezing process ($p>0.05$). The slight increase in the values of Young's modulus after freezing can be attributed to increased crystallinity of the fibrous scaffolds [16, 17]. PCL has a glass transition temperature of -60 ºC and it is possible that after the processes of freezing and thawing that the crystallinity level of the fibers was changed and the ratio between amorphous and crystalline regions altered [2, 8]. Nevertheless, further physicochemical characterization has to be performed in order to quantify the aforementioned properties and relate them to the influence of the freezing process to the fibers.

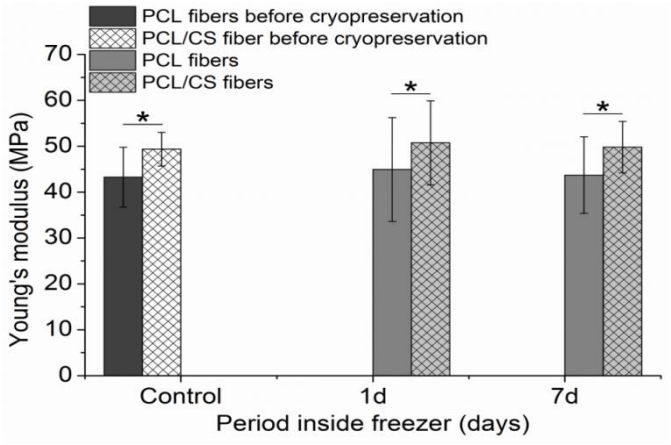

Fig 3: Mechanical properties of electrospun PCL and PCL/CS before cryopreservation and after 1d and 7d inside the freezer (T=-150 ºC); n=5, mean±SD, (*=p<0.05).

CONCLUSION:

The obtained data will allow further research on bio-artificial scaffolds with seeded cells that could be preserved under low temperatures for prolonged periods of time and would be appropriate candidates for regenerative medicine applications.

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