Thermo-responsive scaffolds for stem cell culture

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This thesis aims at the development of scaffolds to allow of cells from the surface by temperature decrease. An initial strategy the surface of the scaffold has been modified with a thermo-sensitive polymer. In a second approach the structure of the scaffold is degraded irreversibly by temperature. The use of thermo-responsive polymers on the culture of stem cells is an alternative to using the enzymatic treatments, which often cause the loss of function and deterioration of the tissues. Were produced by electrospinning nanofibers of polycaprolactone (PCL) with diameters of 500±20nm, which were later modified with thermo-sensitive polymer produced by reversibly addition-fragmentation chain polymerization of the monomer 2-(2'-methoxyethoxy)ethyl-methacrylate (MEO_2MA). The thermo-sensitive scaffolds were obtained by functionalization of the nanofibers of polycaprolactone with the polymer, $p(MEO_2MA)$, whose low critical solution temperature is 27°C. The second type of scaffold was produced by electrospinning from a mixture of polycaprolactone with $p(MEO_2MA)$ in various proportions, giving rise to the thermo-degradable scaffolds. The culture of mesenchymal stem cells with the scaffold produced allowed the study of adhesion proliferation and cell release. Promising results were obtained in terms of cell proliferation for thermo-sensitive nanofibers scaffolds, and in terms of cellular release by temperature on thermo-degradable scaffolds, with an average cell release rate of 50% after two trials, and a superior cell release rate to enzymatic treatment method for the same scaffolds.

Keywords: Polycaprolactone; Electrospinning; 2-(2'-methoxyethoxy)ethyl-methacrylate (MEO_2MA); Mesenchymal stem cells; Thermo-responsive scaffolds; Thermo-degradable scaffolds.

1. Introduction

Stem cells are undifferentiated cells that have the ability to reproduce and differentiate into different types of cells. These cells are responsible for cell growth and maintenance of body tissues, repairing the tissues by a process called self-renewal. The differentiation potential of a stem cell is classified according to the number of different types of cells that it can originate. They are classified as totipotent when they have the ability to originate all cell types of an organism; pluripotent when they have the capacity to develop the most tissues of an organism except the placental tissues; and multipotent when they have the ability to form all the cells of a specific tissue.[1,2,3,4] Stem cells can be found and collected in an undifferentiated state in different tissues of an organism. Are known three types of stem cells, known as embryonic when being collected from the blastocyst of fertilized egg, fetal when collected from fetal tissues and adult stem cells when collected from the patient's/donor tissue. [1,2,3,4] The extracellular matrix is a component of the tissue that provides surface to stem cells adherence, migration and proliferation. The extracellular matrix provides physical support, nutrients and molecules important for cell survival, playing an important role in stem cell differentiation into specific types of tissues or specialized cells. The extracellular matrix is a component of the tissue that provides surface to stem cells adherence, migration and proliferation. The extracellular matrix provides physical support, nutrients and molecules important for cell survival, playing an important role in stem cell differentiation into specific types of tissues or specialized cells. The extracellular matrix is composed by endothelial cells, structural proteins such as collagen type I and IV, laminin, fibronectin, hyaluronic acid and glycosaminoglycan's (GAGs).[1,4,5] Due to the limited number of stem cells that can be collected from the human body and their high demand for research and clinical trials, the need for expansion of stem cells has increased. To obtain a large number of stem cells from a smaller quantity, they need to be grown in appropriate media to support cell growth factors, and an adhesion surface that mimics the extracellular matrix. When these conditions are verified cells have the capacity to adhere and proliferate. Stem cells and some tissues are usually grown in flasks or plates specific for culture, calling up static culture methods. Using bioreactors, dynamic culture methods, most of the problems that static culture methods had was overtaken like the limited transport of nutrients and metabolites, oxygen diffusion and pH control. After the culture of stem cells, the recovery of cell extracts from the surfaces where they grew up or differentiated (bioreactors, culture plates or scaffolds) is usually performed by proteolytic enzymes.[6,7,8] The scaffolds were developed to mimic

the structural function of the extracellular matrix, providing adequate support for the stem cells to adhere, grow and form tissue. Scaffolds act as temporary structure for cell growth and differentiation, they should be designed and developed to be capable of spreading growth factors, adhesion molecules, promoting cellular interactions and present physical properties such as flexibility, hardness or resistance for tissue formation and implementation in-vivo. The biocompatibility and degradation of the materials that comprises the scaffolds are one of the main aspects during its development. They shouldn't release toxic compounds when incubated with biological fluids, and must be compatible with cell support by active or passive interaction with the cells. The porosity and pore interconnectivity of the scaffolds allow the essential interactions between these cells and a good diffusion of molecules needed for supporting their growth.[1,2,3,7,9] Nanofibers have recently been implemented as materials for tissue engineering scaffolds for expansion and differentiation of stem cells. The main advantages of using nanofiber scaffolds in the growth of stem cells are their large surface area relative to, porosity, mechanical properties and easy production and functionalization.[10,11,12] The method most used in the production of nanofibers is electrospinning. This technique produces nanofibers with great reproducibility with a simple equipment apparatus and good control of its functional parameters. [10,11,12] Smart polymers have the ability to change their chain conformation in solution by a physical, chemical or biological stimulus and return to its original conformation when the initial conditions are restored. [8,9,13,14] These polymers can respond to a stimulus by changing its secondary structure by the hydrophilic and hydrophobic balance, its solubility in solution and its intermolecular association. The behavior demonstrated by smart polymers led to its application in microfluidic devices, drug delivery, bioadhesion and actuators. [8,9,13,14] Thermo-responsive polymers are the most studied, being used mostly in surface modification and drug delivery applications. [8,9,13,14] The collapse and expansion of the chains are characterized by a volume phase transition at the low critical solution temperature (LCST) of the polymer, the temperature at which the polymer chains collapse in solution, is due to the formation/disruption of intramolecular electrostatic interactions, hydrophobic interactions and hydrogen bonds.[8,9,13,14] 2-(2'-methoxyethoxy)ethyl-methacrylate (MEO_2MA) is a polymer composed in its chain by methacrylate monomers and macromonomers of polyethylene glycol (PEG) in its side chains. It has a LCST near the body temperature with a phase transition temperature of 26°C, that allows the application of this polymer with biological systems.[16] The temperature response of these type of polymers depend on the size of the ethylene oxide side chains and the balance between the hydrophilic and hydrophobic portions of the molecular structure of the polymer.[15,16]

2. Materials and methods

2.1. Thermo-sensitive nanofiber scaffolds

PCL solutions. For the production of aligned polycaprolactone nanofibers, were prepared 6% (w/w) solutions of polycaprolactone (Sigma, molecular weight 70000-90000) in 1,1,1,3,3,3-hexafluoro-2-propanol (HFP, Sigma), whereas for the random nanofibers polycaprolactone was prepared a solution of 4% (w/w) polycaprolactone (Sigma, molecular weight 70000-90000) em1,1,1,3,3,3- hexafluoro-2-propanol (HFP, Sigma). Allowing the solutions to dissolve and homogenize overnight by mixing.

Nanofiber scaffolds production. The electrospinning equipment used for nanofiber production consists in a high voltage supply (Glassman High Voltage, Inc., EL Series, Model PS/ EL40P01), a syringe pump (KDS Scientific, Model Legato KDS 210), a Teflon tube that connects the polymer solutions of syringe to the needle (EFD International, Inc., needle tip with release valve) with a diameter of 0.84mm, syringes (VWR, Henke Sass Wolf), two aluminum plates and a circular copper plate. For production of aligned nanofibers it was used two parallel rectangular stainless steel plates as collector, with a distance of 3 cm between them and as default parameters a fixed flow rate of the polymer solution of 1 mL/h, a distance between needle and plates of 20 cm and an electric potential of 26kV. For random nanofibers production it was used a circular copper plate collector, being used as default parameters a fixed flow rate of the polymer solution of 0.8mL/h, a distance between the needle and plates of 25cm and an electric potential of 20kV. After deposition the nanofibers were allowed to stand for 2 hours for proper drying before proceeding to the fixation in

circular glass coverslips with biocompatible adhesive glue (Silastic Silicone-adhesive medical, type A). After the fixation step of the different types of nanofibers it was allowed to dry for 12 hours, using a scalpel after this period to collect the nanofiber scaffolds of both collectors.

Polycaprolactone nanofiber scaffolds functionalization. To perform this procedure was prepared an ethanol solution (Fisher)/water (1/1, v/v) and a 10% solution of 1,6-diaminohexane in isopropanol (Fisher Chemical), allowing homogenization by magnetic stirring overnight. After preparation of the solutions, scaffolds were placed in well plates and immersed for two hours in ethanol/water by gentle agitation, been then washed with distilled water. Scaffolds were immersed in isopropanol for two minutes to remove water excess, adding after 10% 1,6-diaminohexane and incubating at 37°C for three hours. After the incubation time the aminolysis solution was removed and added once distilled water for 5 minutes to remove unreacted 1,6-diaminohexane. After that, water was removed from scaffolds and then is added distilled water again, letting in light agitation for 24 hours. The supernatant is removed from the wells and scaffolds are allowed to dry for 24 hours in a desiccator under vacuum. The volume used for immersion of the scaffolds was 300µL in all solutions.

Thermo-sensitive polymers p(MEO_2MA) and p(MEO_2MA-co-NAS). In this part of the work was intended to synthesize by reversible addition-fragmentation polymerization (RAFT), the thermo-sensitive polymer p(MEO2MA) and the p(MEO_2MA-co-NAS) with the reactive monomer N-Acryloxysuccinimide (NAS), which has an activated ester derivative that allows the functionalization of the polymer with fluorescent molecules and other compounds of interest. The desired number of monomers per chain is given by the ratio [M]/[RAFT Agent+ Initiator], being chosen a ratio of 600 that indicates the desired number of monomers per chain, it was defined a ratio [initiator]/[CTA] of [1/10] and for the copolymer MEO_2MA-co-NAS it was defined a percentage of NAS of 2%. For the synthesis of the polymer MEO2MA with 600 monomers is weighed in a volumetric flask 1 mg of AIBN, 24,58mg of RAFT agent and 7,565g of MEO_2MA. After addition of the compounds is added a magnetic stirrer and the flask is sealed, creating an atmosphere of inert argon gas and starting the homogenization of the mixture at room temperature for 15 minutes. After the homogenization time is added to the mixture 17,9mL of Isopropanol, letting the mixture to homogenize under argon over 10 minutes. After the homogenization time the oil bath is set to 90°C which initiates the polymerization, being placed in the cover a plastic balloon filled with argon to allow the balance of gases, for 24-36 hours. After this period, the bath is set to 60°C to ensure the reaction has stopped and that this is hot enough to be able to precipitate. With a pipette, the polymer is precipitated in ice-cold ether, observing the formation of two-phases in solution. The ether solution was renewed for three consecutive days, always observing two phases. After the washing, the supernatant is removed, allowing drying the precipitate which corresponds to the polymer MEO_2MA. For the synthesis of the copolymer MEO_2MA co-NAS with 600 monomers is weighed in a volumetric flask 1 mg of AIBN, 24,58mg of RAFT agent, 7,414g of MEO_2MA and 135,95mg of NAS. After addition of the compounds a magnetic stirrer is added and the flask is sealed, creating an atmosphere of inert argon gas and starting the homogenization of the mixture at room temperature for 30 minutes. After the homogenization time is added 14,84mL of dried dimethylformaldehyde (DMF) to the mixture, allowing the mixture to homogenize under argon over 20 minutes. After the homogenization time the oil bath is set to 90°C to initiate polymerization, repeating the steps made in the MEO_2MA polymer synthesis.

Thermo-sensitive nanofiber scaffolds. To produce nanofibers functionalized with thermo-sensitive polymer MEO_2MA, first has to be prepared a polymer solution in which the concentration is variable according to the number of scaffolds that need to be functionalized. Is used 50mg of polymer and 1 ml of dry DMSO (Sigma) per sample dissolving by stirring in an argon atmosphere. When the solution is homogenized is added 17µL of activating agent N-(3-dimethyl aminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) per sample, mixing by stirring in an argon atmosphere. Then, the functionalized polycaprolactone nanofiber scaffolds are placed in vials, sealed in an argon atmosphere for 5 minutes. After this time 1 ml of the polymer solution is removed and added directly on the scaffold, leaving it immersed, repeating the same

process for the remaining scaffolds. When all of the polymer scaffolds are immersed in solution they are placed to incubate for 48 hours at room temperature on rotatory shaking at 300 rpm. When the incubation time exceeded, polymer solution is withdrew and is added 2 mL of a solution of 50% (v/v) isopropanol in water, allowing it to stir at 300rpm for 1 hour. After one hour the isopropanol/water solution is removed and is added 3mL of water, stirring for 1 hour. After the washing steps the scaffolds are placed in a desiccator for drying under vacuum for 24 hours.

2.2. Thermo-degradable MEO_2MA/PCL scaffolds

Preparation of polymer blend solutions. For the synthesis of thermo-degradable scaffolds were prepared a 20% solution (w/w) of polycaprolactone (Sigma, molecular weight 70000-90000) with the polymer produced MEO2MA in the solvent 1,1,1,3,3,3 hexafluoro-2-propanol (HFP, Sigma) at different proportions. To study these scaffolds were prepared mixing solutions of 90%MEO_2MA /10% PCL, 75%MEO_2MA/25%PCL and 50%MEO_2MA/ 50%PCL, wherein the mixing of the two amounts preforms 20% (w/w) in HFP. The solution is mixed in gentle agitation for 24 hours at room temperature.

Thermo-degradable scaffolds. Prior to performing the electrospinning, the solutions were placed on the vortex mixer for 10 minutes to ensure that the solution was homogenized. For all solutions the parameters synthesis was fixed by using a circular collector of copper, an electrical potential of 25 kV, a distance of 25cm between the collector and the needle and a flow rate of 1.5 ml/h. As variable parameters of synthesis it had a temperature in the range of 15-25°C and a relative humidity of 25-50%. In this approach the circular glass slides (VWR, 32 mm diameter) are first placed on top of the collector, and deposition is made directly on the coverslips which serve as a substrate for scaffolds. After drying, with the help of a needle the biocompatible glue was spread in the edges of the glass coverslips with the thermo-degradable scaffolds. After the fixation step were left to dry for 12 hours, using a scalpel to collect the scaffolds of the collector. Then the samples are placed in a desiccator under vacuum for 24 hours.

Mesenchymal stem cells culture. To carry out this study, the produced scaffolds were immobilized on culture well plates of low cell adherence (Corning) with biocompatible glue. When cells are ready, the scaffolds are incubated in antibiotic solution (Invitrogen) for 3 hours at 37°C. After three hours of incubation the antibiotic was withdrew and cells were cultivated (10000-30000 per scaffold). Were used as culture media DMEM (Life Technologies) supplemented with 10% fetal bovine serum, Xenofree (Life Technologies) formulated for mesenchymal cell culture and Xenofree with cell start (Life technologies) with offers adhesion proteins and cell growth factors to the scaffold, being incubated for 1 hour at 37°C before the addiction of Xenofree. The culture media were added at a temperature of 37°C after 30 minutes of pre-culture with MSCs, allowing them to adhere to the scaffolds before addition of culture medium. The plates were placed in an incubator at 37°C under a humidified atmosphere of 5% CO_2.

Alamar blue cell quantification. First was prepared a calibration curve that relates a specific number of cells with the fluorescence read at same culture conditions that will be done with the scaffolds. After that the quantification of cells present in the thermo-sensitive scaffolds and the thermo-degradable scaffolds was held by adding to culture medium 100µL of Alamar Blue. That creates a solution in the well of 1% (v/v) of Alamar Blue, incubating for 2 hours at 37°C in a humidified incubator with an atmosphere of 5% CO_2. The fluorescence intensity of the Alamar Blue solution with cell medium was measured using a microplate reader, an excitation wavelength of 560 nm and emission of 590 nm.

Temperature cell release of scaffolds. To perform these tests was added to the culture medium of the scaffolds 1 ml of fresh IMDM, which lower quickly the temperature of the medium, placing after the plate in an ice bed to maintain the low temperature for 1 hour. During this period the plates were agitated every ten minutes for 2 minutes. After one hour the culture media is centrifuged for 7 minutes at 1250rpm in Falcon tubes. After centrifugation the supernatant is removed the precipitate was solubilized in a volume of 150µL. The mesenchymal cells that were efficiently released by the temperature in the scaffolds were counted. For this are placed 10µL of duplicate samples of each scaffold cell solution in a 96 well plate and stained with 10µL of Trypan blue. The solutions were homogenized in each

well and placed after in a hemocytometer. The cell count is performed with the help of an optical microscope and was carried out a calculation to know the number of cells present in each scaffold. After the counting scaffolds were autoclaved and destroyed.

3. Results and discussion

3.1. Thermo-sensitive scaffolds

SEM characterization of polycaprolactone nanofibers. SEM was used to characterize the scaffolds and the frequency of diameters was analyzed using the software ImageJ. 100 nanofibers were examined using an average of three measurements for nanofiber**.** It was measured an average diameter of 495±20nm in random nanofibers and 500±20nm average diameter for aligned nanofibers.

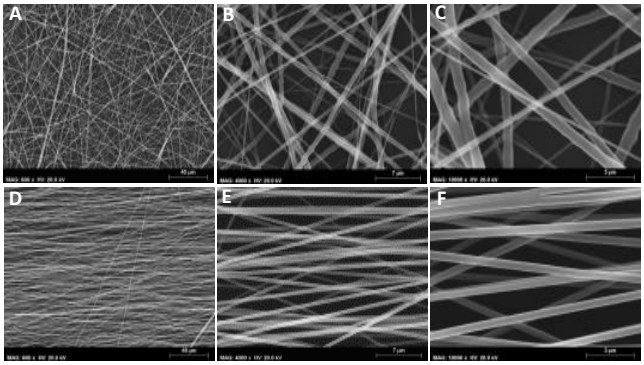

Figure 1 - SEM images of random and aligned polycaprolactone nanofibers prepared from 4% and 6% PCL solution in HFP. A,B,C) Random polycaprolactone nanofibers at 270×270µm, 50×50µm e 15×15µm scales respectively. D,E,F) Aligned polycaprolactone nanofibers at 270×270µm, 50×50µm e 15×15µm scales respectively.

The aligned scaffolds showed a fairly uniform nanofiber diameter distribution, compared with the random scaffolds where a broader diameter distribution is observed. This difference between the scaffolds is a common result, since the collectors used have different geometries that create different electrical fields.

MEO_2MA and MEO_2MA-co-NAS characterization. Analyzing the results obtained by Gel Permeation Chromatography the polymer p(MEO_2MA), has a low polydispersity Mw/Mn=1.23, with a number average molecular weight Mn=(71±0.9)×10^3g/mole and a mass average molecular weight Mw=(89±0.8)×10^3g/mole. Making some calculations it was possible to reach to the experimental 370 monomers per chain of the produced polymer. This result was lower than the initially expected (600 monomers/chain), meaning that was obtained an incomplete polymerization reaction (presence of oxygen or low conversion ratio of the monomers).

The characterization of p(MEO_2MA) and p(MEO_2MA-co-NAS) in respect to their lower critical solution temperatures (LCST) was made by turbidimetry (figure 2). To determine the LCST of the polymers MEO_2MA (A) and MEO_2MA-co-NAS (B) were calculated the intersections of the adjustment lines (figure 2), at 25°C-28.5°C (A) and 25.7°C-30.1°C (B). The average transmittance value at these temperatures corresponds to an LCST of 26.7°C for MEO_2MA and 27.9°C for MEO_2MA-co-NAS.

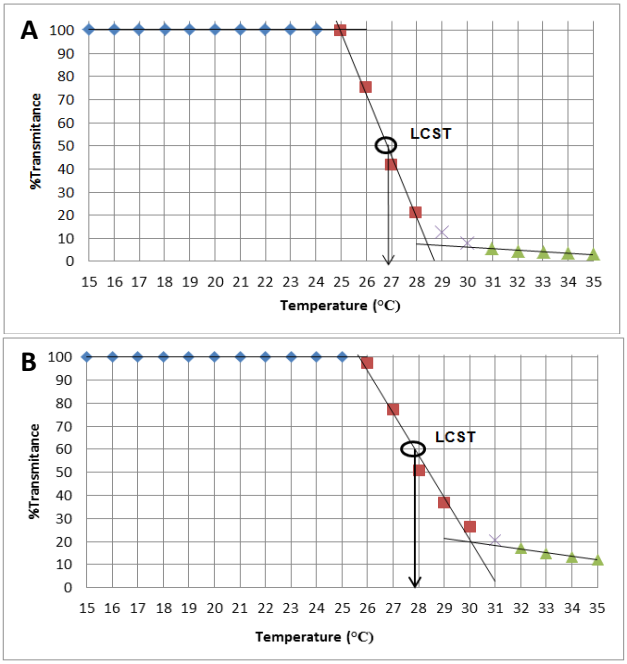

Figure 2 - % Transmittance vs. Temperature graph of polymer MEO2MA (A) and MEO2MA-co-NAS (B) in a solution of 1mg/ml at a wavelength of 650nm and a heating ramp of 1°C/min in a range of 15°-35°C. Characterization LCST using linear equations in different temperature ranges (blue, red and green).

Thermo-sensitive scaffolds characterization. The polymer coverage of the fibers was estimated by colocalization using Laser scanning confocal microscopy. The polycaprolactone nanofibers were labeled with Cy5 and the p(MEO_2MA-co-NAS) was labeled with Lucifer Yellow (figure 3).

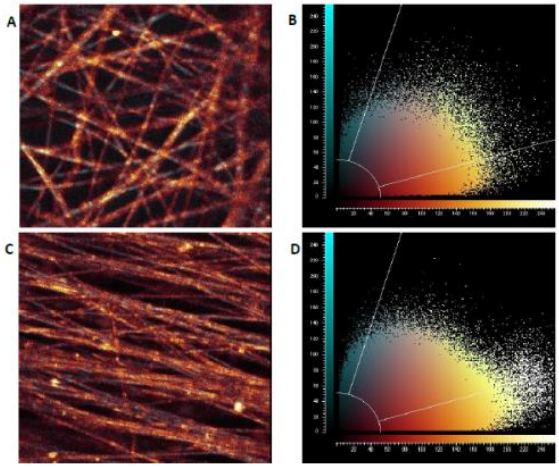

Figure 3 - Images obtained by confocal laser microscopy of the random (A) and aligned (C) polycaprolactone nanofibers marked by Cy5 and covered with marked polymer MEO_2MA-Ly. A,C) Images obtained by the overlap of the two emissions simultaneously. B,D) Graphics with the results of the characterization by colocalization of the images on the left.

In the images resulting from the overlap of the two fluorescence images (fig.3 –A,C) is possible to observe good coverage of the nanofibers. The inner part of the white lines of the colocalization graph (fig.3 – B,D) represent the overlaps in the same pixel of two fluorescent emissions, outside the lines are the emissions without overlap. When at the same pixel overlaps two fluorescent emission the result is the sum of the two color emissions observed in the figure 3-A and 3-C. This result shows that the nanofibers exhibit a high coverage density by p(MEO_2MA).

The characterization of aligned and random thermo-sensitive scaffolds by SEM (figure 4), was used to study the morphology of the PCL nanofibers after functionalization with thermo-sensitive p(MEO_2MA). The nanofiber surface was clearly changed when compared with the nanofibers without polymer. The increase roughness is an indicator that they are covered by polymer and the functionalization of nanofibers with the thermo-sensitive polymer was efficient.

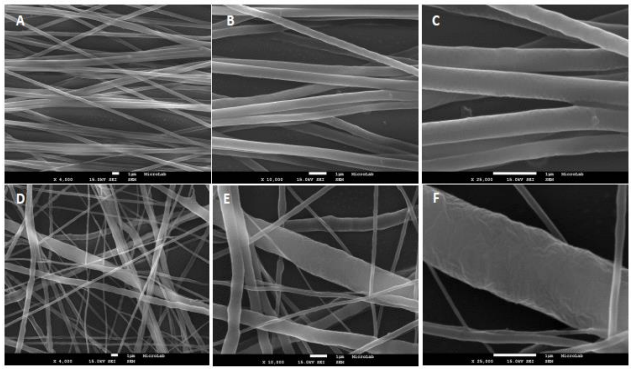

Figure 4 - Images obtained by SEM for the characterization of the morphology of aligned and random nanofibers of polycaprolactone covered with polymer MEO_2MA. A, B and C) Nanofibers of polycaprolactone aligned with MEO_2MA at 45×45µm, 15×15µm and 5×5µm scales respectively. D, E and F) Polycaprolactone random nanofibers with MEO_2MA at 45×45µm, 15×15µm and 5×5µm scales respectively.

The thermo-sensitive scaffolds were tested for changes of the surface by temperature stimulus in figure 5. Scaffolds were immersed in cold water (10°C), dried and characterized by AFM. Repeated in hot water (50°C). The surface of the thermo-sensitive nanofibers changed with the temperature of the medium. This fact is indicative of the presence of the polymer MEO_2MA, by the change in the conformation of the chains that cause surface modification.

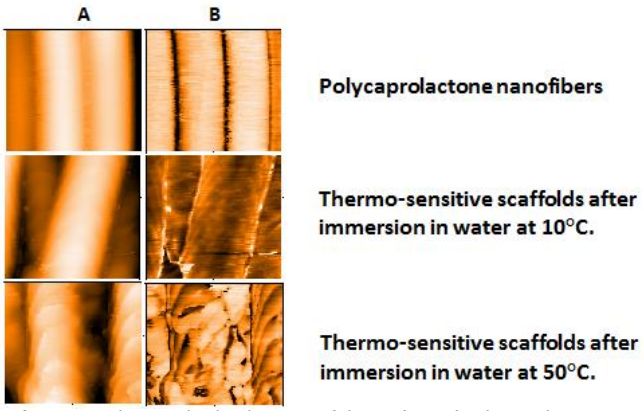

Figure 5 - Characterization by AFM of the surface of polycaprolactone nanofibers covered with MEO_2MA by temperature change (2x2µm scale for each image). A) Characterization of the surface by amplitude. B) Characterization of the surface by phase.

3.2. Thermo-degradable scaffolds

Thermo-degradable scaffolds of 90%MEO_2MA/ 10%PCL, 75%MEO_2MA/25%PCL and 50%MEO_2MA/ 50% PCL were produced and characterized. In figure 6, characterization images of a thermo-degradable scaffold of 90% MEO_2MA/10%PCL by SEM and AFM were shown after been produced by electrospinning. SEM and AFM images show that the surface is flat, with some irregularities, which may be indicative of the existence of some sort of internal structure on the film. Then was performed a degradation test in these scaffolds in order to simulate the conditions in cell culture and release tests. Scaffolds were incubated in water at 37°C for 1, 4 and 7 days, for stability tests and placed in cold water at 10°C for one and two hours with gentle shaking, placing immediately in a desiccator. It was observed that the rate of degradation of these scaffolds was constant over the days of incubation, and heat incubation does not influence the rate of degradation of the scaffolds with cold water. It was also observed that the structure of the scaffold was maintained throughout the seven days of incubation at 37°C.

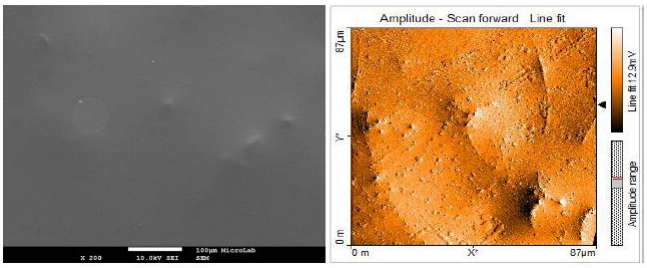

Figure 6 - SEM characterization on the left at 500×500µm and AFM characterization on the right at 87×87µm scale, to study the structure and topography of the thermo-degradable scaffold of 90%MEO_2MA/10%PCL.

The stability and degradation tests of the 90% MEO_2MA/10%PCL thermo-degradable scaffolds by SEM are shown in figure 7. It was observed that the structure of the scaffolds did not change with the incubation time of 7 days at 37°C. By lowering the temperature, it was observed that the scaffold structure was completely degraded after immersion in cold water at 10°C and there is a greater degradation of the scaffolds after two hours of immersion in cold water, when compared with the results after one hour.

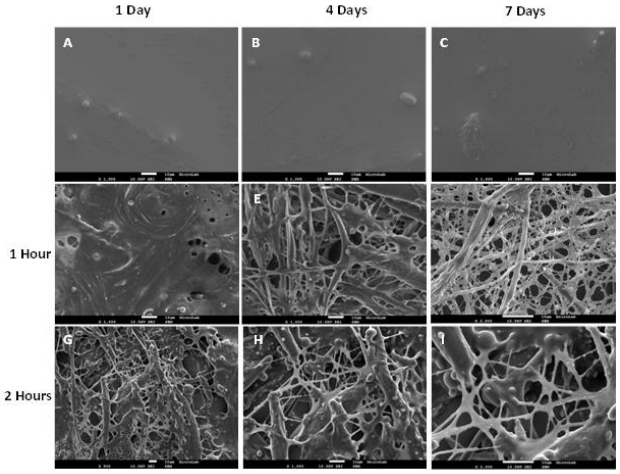

Figure 7 – SEM characterization of stability and degradation of 90%MEO_2MA/10%PCL thermo-degradable scaffold. A, B and C) 90%MEO_2MA/10%PCL scaffold after 1, 4 and 7 days in water at 37°C to 95×95µm scale respectively. D, E and F) 90%MEO_2MA/10%PCL scaffold after 1 hour in water at 10°C to 140×140µm, 140×140µm and 50×50µm scale respectively. G, H and I) 90%MEO_2MA/10%PCL scaffold after 2 hours in water at 10°C to 200×200µm, 140×140µm and 50×50µm scale respectively.

SEM and AFM in figure 8, of the thermo degradable scaffolds of 75%MEO_2MA/25%PCL, after production by electrospinning. The surface of this type of scaffold is porous and rough, appearing to be a porous polymer film with structures that appear to be fibers covered by polymer.

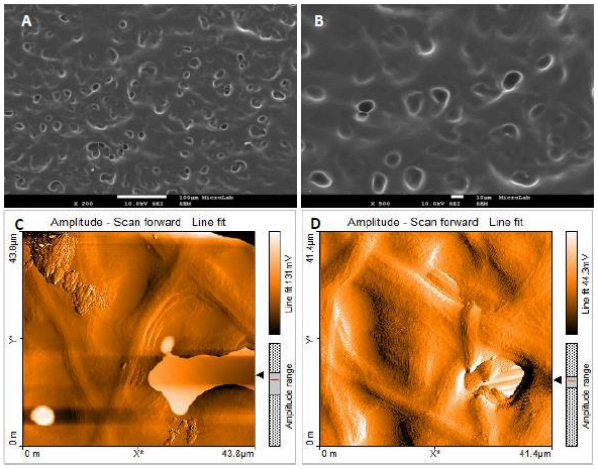

Figure 8 - Surface characterization of the thermo-degradable scaffolds of 75%MEO_2MA/25%PCL by SEM and AFM. A and B) Surface of the 75%MEO_2MA/25%PCL scaffold to 500×500µm scale. C and D) Scaffold surface characterization by AFM related to its amplitude.

Stability and degradation tests were made in the 75% MEO_2MA/25%PCL scaffolds, and characterization by SEM (figure 9). Which showed that scaffolds did not change in the 7 day incubation period at 37°C. By decreasing the temperature can be observed that the scaffold structure has been partially degraded after immersion in cold water, a greater degradation was observed after two hours, when compared with the results after one hour.

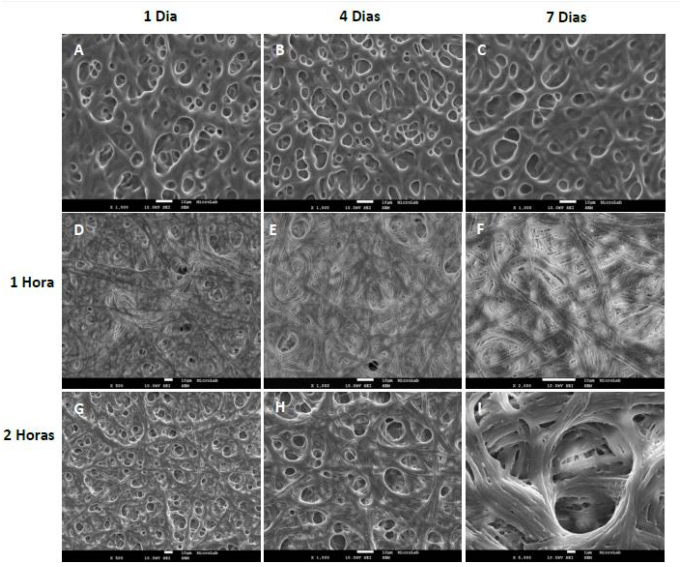

Figure 9 - Characterization of the degradation and stability of the 75%MEO_2MA/25%PCL film by SEM. A, B and C) 75%MEO_2MA/25%PCL Scaffold after 1, 4 and 7 days in water at 37°C to 90×90µm scales. D, E and F) 75%MEO_2MA/25%PCL scaffold after 1 hour in water at 10°C to 200×200µm, 100×100µm e 50×50µm scales respectively. G, H and I) 75%MEO_2MA/25%PCL scaffold after 2 hours in water at 10°C to 200×200µm, 100×100µm e 20×20µm scales respectively.

SEM and AFM images of the thermo-degradable scaffolds of 50%MEO_2MA/50%PCL characterization regarding its structure after production by electrospinning (figure 10). The produced fibers had

diameters close to 1 µm, cylindrical form and smooth surface.

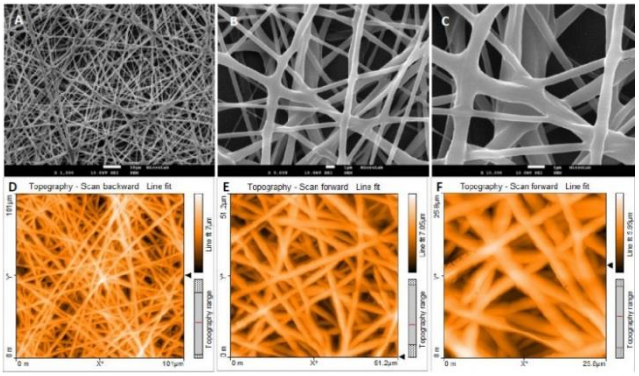

Figure 10 –Surface characterization of the thermo-degradable scaffold of 50%MEO_2MA/50%PCL by SEM and AFM. A, B and C) 50%MEO_2MA/50%PCL scaffold to 110×110µm, 22×22µm and 11×11µm respectively. D, E and F) Characterization of the surface of 50%MEO_2MA/50%PCL scaffold by topography at different scales.

Thermo-degradable scaffolds of 50%MEO_2MA/50%PCL were characterized by SEM after the stability and degradation tests (figure 11). it was observed that the structure has not changed with the incubation time of 7 days at 37°C. Regarding the degradation of these scaffolds for decreased temperature may be noted that the structure of the scaffold remains intact after immersion them in cold water, observing only a superficial degradation by the appearance of cavities in the fibers of the scaffold after one and two hours of degradation.

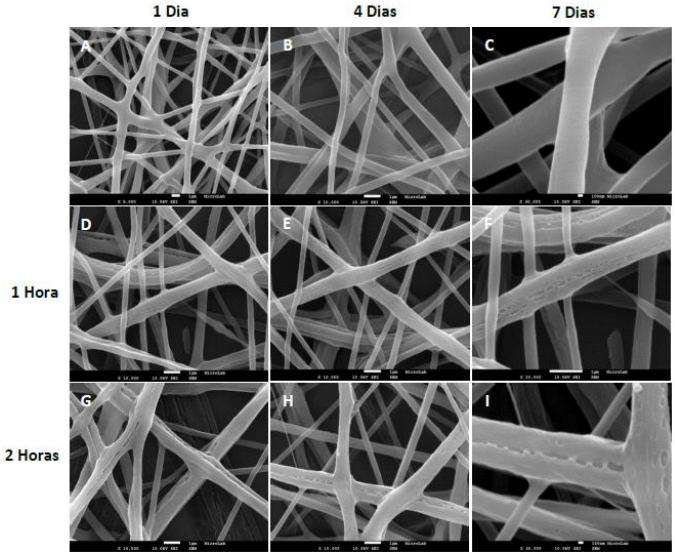

Figure 11 - Characterization of the degradation and stability of the 50%MEO_2MA/50%PCL scaffold by SEM. A) 50%MEO_2MA/50%PCL scaffold after 1. 4 and 7 days in water at 37°C to 18×18µm, 10×10µm and 4×4µm scales respectively. D, E and F) 50%MEO_2MA/50%PCL scaffold after 1 hour in water at 10°C to10×10µm and 5×5µm scales respectively. G, H and I) 50%MEO_2MA/50%PCL scaffolds after 2 hours in water at 10°C to 10×10µm and 4×4µm scales respectively.

3.3. Proliferation and cell release

Mesenchymal stem cells in thermo-sensitive scaffolds. 10000 mesenchymal stem cells were cultured in triplicates of thermo-sensitive random scaffolds in DMEM+10%FBS, Xenofree and Xenofree with cell start for 9 days medias, performing studies of cell proliferation and release at days 7 and 9 of the culture time (figure 12).

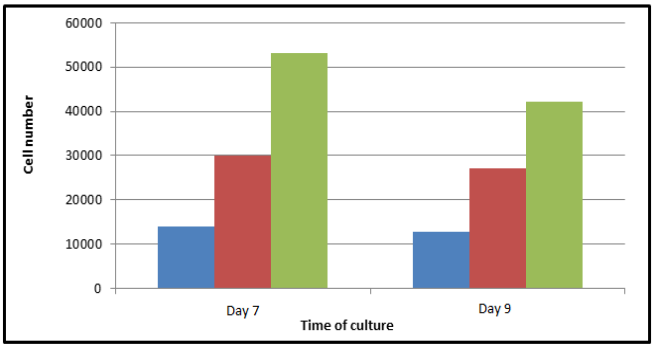

Figure 12 - Graph with the number of cells counted by Alamar blue in random thermo-sensitive scaffolds for day 7 and 9 in DMEM+10%FBS culture medium (blue), Xenofree (red), and Xenofree with of cell start (green).

Concluding on the proliferation assay performed on thermo-sensitive random scaffolds, it can be observed good indicators of cell adhesion and growth over the culture time. Since the average adhesion in polycaprolactone scaffolds is between 10 and 30%, and the thermo-sensitive scaffolds were first cultivated with 10,000 cells, at best adhered to the scaffolds 3000 cells. Looking at cell densities obtained after 7 days, was observed for Xenofree a theoretical cell multiplication of 10, and in Xenofree media with cell start a theoretical cell multiplication of 17.6. Were performed a cellular release test on these scaffolds, by decreasing the temperature of the media to 10°C for 1 hour, not been achieved a significant release of cells from scaffolds. Were performed a culture of 10000 mesenchymal cells in triplicates on aligned thermo-sensitive scaffolds in DMEM+10%FBS, Xenofree and Xenofree with cell start for 9 days, performing cell proliferation and release studies in days1, 4 and 9 of culture time, figure 13.

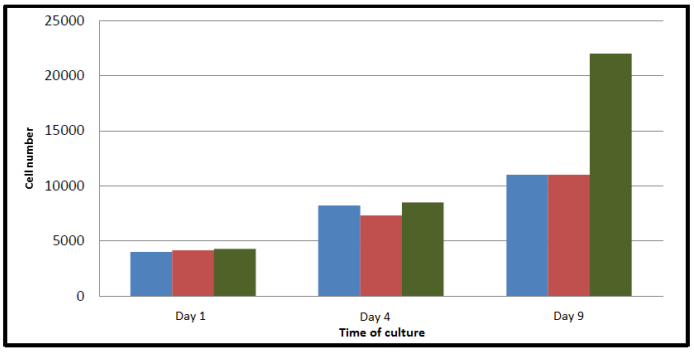

Figure 13 - Representative chart of cell proliferation on aligned thermo-sensitive nanofibers during 9 days for the culture medias DMEM+10%FBS in blue, Xenofree in red and Xenofree with cellular initiation in green.

The observed cell multiplication on aligned thermo-sensitive scaffolds, it was achieved after 9 days of culture a fold increase of 3 for scaffolds cultured in DMEM+10%FBS and Xenofree, and a fold increase of 5.5 for scaffolds cultured in Xenofree with cell start. Aligned thermo-sensitive scaffolds have reported lower proliferation compared to random thermo-sensitive scaffolds. This result tells us that Mesenchymal stem cells need to grow in an extracellular matrix structure quite disorganized. Were performed a cellular release test on these scaffolds, by decreasing temperature of the medium to 10°C for 1 hour, not been achieved a significant cell release on this scaffolds.

Mesenchymal stem cells in thermo-degradable scaffolds.

A cell proliferation test and cell release was performed with thermo-degradable scaffolds 75%MEO_2MA/ 25%PCL, in the culture medias DMEM+10%FBS and Xenofree for 7 days, retrieving results at days 1 , 4 and 7 (figure 14).

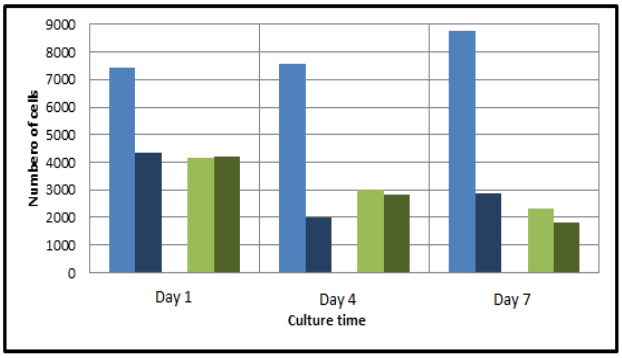

Figure 14 - Representative chart of cell proliferation (light colors) vs. cell release (dark colors)on thermo-degradable scaffolds during 7 days for the culture medias DMEM+10%FBS in blue, Xenofree in green.

The cell release results in the thermo-degradable scaffolds of 75%MEO_2MA/25%PCL, were successful in terms of cell release, with a 60% average of release in the trial. A limiting factor of the results was obtained on cell death in the proliferation assays, where the cells not developed properly in the scaffolds, and could have influenced directly the result of cellular release.

A test of cell proliferation and cell release was performed with thermo-degradable scaffolds of 90%MEO_2MA/10%PCL, 75%MEO_2MA/ 25%PCL scaffolds and random nanofibers scaffolds of polycaprolactone (controls) were cultured with 30000 mesenchymal stem cells. The medium used was Xenofree. Were used standard enzymatic release methods and temperature release, to establish a comparison between the two in terms of efficiency. The thermo-degradable scaffolds and controls were cultured for 7 days, performing tests on days 1, 4 and 7 days (figure 15).

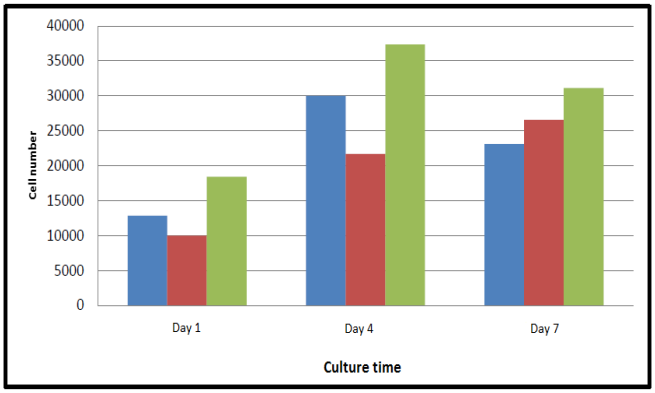

Figure 15 - Graph of cellular proliferation on thermo-degradable scaffolds of 75%MEO_2MA/25%PCL in blue, 90%MEO_2MA/10% PCL scaffolds in red and controls (PCL scaffolds) in green after 7 days in Xenofree culture media.

For cell proliferation it was obtained a fold increase of 1.85 in the thermo-degradable scaffolds of 75%MEO_2MA/25%PCL, 2.7 fold increase on thermo-degradable scaffolds of 90%MEO_2MA/10%PCL and 1.7 fold increase in controls of polycaprolactone scaffolds. This means that both thermo-degradable scaffolds owned a fold increase rate higher than polycaprolactone scaffolds, being achieved the best results for thermo-degradable scaffolds of 90%MEO_2MA/10%PCL. For this experiment was observed on thermo-degradable scaffolds better results in terms of cell proliferation when compared with controls, but less positive compared to the initial cell adhesion of controls. Was performed a cellular release test on these scaffolds, by decreasing the temperature of the medium to 10°C for 1 hour and Accutase for 7 minutes at 37°C (figure 16).

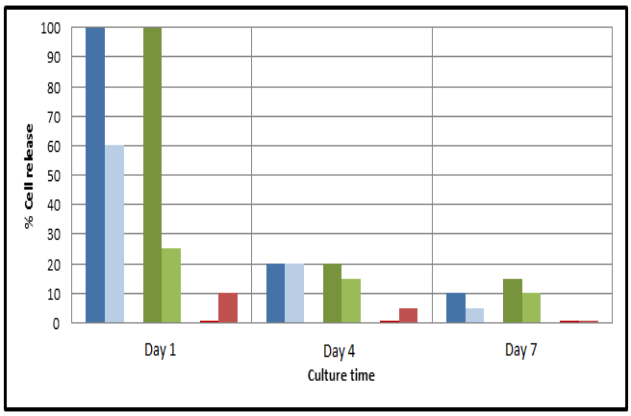

Figure 16 - Graph of the cell release percentage by temperature (dark colors) vs. enzymatic release (light colors) on 75%MEO2MA/25%PCL in blue, 90% MEO_2MA/10%PCL in green and controls (PCL scaffolds) in red.

It was possible to observe cell release by lowering the temperature in both thermo-degradable scaffolds during

the 7 days of culture, while in polycaprolactone controls the cells not freed with temperature as expected. For the enzymatic release method, it was possible to release mesenchymal stem cells in thermo-degradable scaffolds in the 7 days of culture, while in the controls was not achieved cell release after 7 days of culture. Apparently the temperature release methods have been more effective than the enzymatic release method for mesenchymal stem cells. It was also observed that the methods of temperature release and enzyme release, were less effective with increasing time of culture, but the efficiency of release was higher in all cases for the release by temperature with an average of 45%. In the polycaprolactone controls the enzymatic cell release was very limited and the result had an average of 15% cell.

Conclusions

The objective of this work was to prepare and characterize two types of thermo-responsive scaffolds. The results for cell adhesion on the thermo-sensitive scaffolds was better than the adhesion results in the thermo-degradable scaffolds, but lower when compared with controls. The proliferation tests had better results for random thermo-sensitive scaffolds than for thermo-degradable scaffolds and controls, with the better case yielding a 17-fold increase after 7 days of culture. The cell release by temperature tests had better results for thermo-degradable scaffolds than for thermo-sensitive scaffolds, being achieved after two experiments an average cell release of 50%. It was observed better results in cell release by temperature on thermo-degradable scaffolds than for the conventional enzymatic release method, which had an average cell release of 15%. Can be concluded that thermo-sensitive scaffolds are a better alternative for cell proliferation than the polycaprolactone scaffolds. In terms of cell release the results concluded that the temperature method was more effective than the enzymatic method.

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