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Preparation of electrospun nanofibers from solutions of different gelatin types using a benign solvent mixture composed of water/PBS/ethanol.

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ABSTRACT

The feasibility of using Phosphate Buffer Saline (PBS)/ethanol mixtures as a benign solvent to electrospin three types of gelatin was studied. Gelatins with different chemical properties, such as Bloom, were selected and the effect of the gelatin nature and its concentration on the electrospinnability of the dope solution and on the fiber diameter of the electrospun mats were studied. Viscosity of the gelatin solution, which follows a power law relationship with the gelatin concentration, was found to significantly influence the morphology of the mats and the fiber diameter. It was demonstrated that the PBS/ethanol solvent interacted with the gelatins as a good solvent with a Flory exponent of 0.65. In addition, the effect of the solvent composition on the fiber formation process was evaluated corroborating that the ionic strength of the medium and the PBS/ethanol ratio significantly affected the morphology and the diameter of the electrospun fibers. Chemical structure and thermal stability of the electrospun gelatin mats were characterized by Fourier Transform Infrared Spectroscopy (FTIR) and Differential Scanning Calorimetry (DSC). Finally, cytotoxicity of the electrospun mats was analyzed by the Alamar Blue assay, using human foreskin fibroblasts (BJ-5ta), resulting in a high cell viability (80-90%) regardless the type of gelatin.

Keywords: electrospinning, nanofibers, gelatin, Phosphate Buffer Saline, ethanol.

1. INTRODUCTION

Although electrospinning is a quite old technique[1] based on the application of a high voltage electrostatic field between a capillary syringe, containing a polymer solution, and a grounded collector where the polymer fibers are deposited[2-4], in recent years numerous reports have proposed their use for the development of applications in several fields such as biomedical, pharmaceutical, biotechnological and environmental engineering.[5-6] Among all these applications, the design and manufacturing of scaffolds made of electrospun fibers for tissue engineering applications have gained attention due to the capacity of some natural polymers for mimicking the structural and functional properties of extracellular matrices.[7-8] Amid the various natural polymers that could be electrospun into fibrous scaffolds[9-10], gelatin is one of those most extensively investigated since it has similar properties to those of collagen and it is also an abundant and low cost material.

Gelatin is obtained from the parent protein collagen by chemical or biochemical processes that break up the secondary and further structures of the protein through several degrees of hydrolysis of the polypeptide backbone.[11] For industrial gelatin production the raw material may be any collagen-containing tissue such as skin, muscle and bone and, depending on the method of hydrolysis, two different types of gelatin can be produced: type A (obtained by acid treatment) and type B (obtained by alkaline treatment). Consequently, the chemical properties of different gelatins are affected by the animal species they come from, the nature of the original tissue as well as by the type of treatment implemented for their extraction.[12-13] All those factors determine some important parameters such as the exact amino acid composition, the molecular weight distribution and the gel strength of each gelatin which are the key parameters controlling the viscosity of gelatin solutions. Since viscosity is one of the major parameters ruling electrospinnability, the aforementioned parameters are also decisive in the nanofiber production by this technique.[14]

Regardless the type of gelatin, another crucial factor influencing the electrospinning process is the solvent selection.[15] Particularly, in order to successfully electrospin gelatin, the solvent system must be capable of avoiding the gelation process that occurs between gelatin and cold water (< 37 ºC)[16] and inducing an optimal viscosity to the dope solution, facilitating its movement through the syringe during electrospinning. In this regard, some complex solvents such as 1,1,1,3,3,3-hexafluoro-2-propanol or 2,2,2-trifluoroethanol were initially used to dissolve and electrospin natural polymers such as gelatin at room temperature[17-20], but their ability to form strong hydrogen bonds with protein based polymers hinders the complete removal of these solvents from the obtained fibers. As a result, not only the protein chemical structure is affected but undesirable reactions can also occur due to the high cytotoxicity of such solvents.[21] Alternatively, solvents based on carboxylic acids, such as formic acid[22-23] and acetic acid[15,24-26], have been recently proposed for electrospinning of protein-based polymers.[14,27] However, in most of the reported cases, a high concentration of acid (more than 60% v/v) is required to achieve a proper electrospinning and, unfortunately, this fact induces the partial decomposition of gelatin and adversely affects the structural integrity of nanofibers.[22]

In order to overcome the disadvantages of using fluoroalcohols or carboxylic acids, a solvent consisting of a dilution of Phosphate Buffer Saline in ethanol (hereafter water/PBS/ethanol) has already been proposed to prepare collagen or gelatin solutions for electrospinning.[10,28-29] Such mixed solvent effectively dissolves gelatin, disrupting

both hydrophobic and hydrogen bonding interactions between amino acids of gelatin as well as it provides an adequate medium for electrospinning.[30] A suitable balance of the three components of the mixture may change some properties of the dope solution such as viscosity, surface tension, conductivity and degree of gelation[31], therefore determining their electrospinnability.

The purpose of this study was to assess the feasibility of using water/PBS/ethanol mixture as a benign and advantageous solvent for the electrospinning of different gelatins. Specifically, the three studied gelatins were obtained from bovine skin (BS), bovine bone (BB) or porcine skin (PS). BS and BB gelatins are type B gelatin whereas PS gelatin was obtained by acid treatment (type A). In addition, the existing relationships between some physicochemical properties of the dope solution and the characteristics of the obtained fibers were investigated. Particularly, the fiber diameter, the changes of chemical structure due to solvent and the cytotoxic effects were examined.

2. EXPERIMENTAL

2.1 Materials

Gelatin type B from bovine skin (BS) (Bloom ~ 225 g) and gelatin type A from porcine skin (PS) (Bloom ~ 300 g) were purchased in their powder form from Sigma Aldrich (Madrid, Spain). Gelatin type B from bovine bone (BB) (Bloom ~ 250 g) was provided by Rousselot Gelatin S.L (Girona, Spain). All gelatins were used without further treatment or purification. Note that Bloom value is a currently used test to measure the strength of a gelatin and is directly related to molecular weight of the polymer.[32]

Phosphate Buffer Saline (PBS) solution was purchased in tablet form from Sigma Aldrich. One tablet dissolved in 200 mL of deionized water yields 1X PBS solution (0.01 M phosphate buffer, 0.0027M potassium chloride and 0.137 M sodium chloride), with pH 7.34 at 25ºC. Different PBS buffers were obtained by diluting a 20X PBS stock (10 tablets in 100ml) using bidistilled water. To prepare the dope solution, each gelatin was dissolved at room temperature in PBS/ethanol mixtures of different composition.

2.2 Nanofibers mats preparation

Electrospun gelatin nanofibers were obtained by the electrospinning process, performed in a home-made device developed by INTEXTER.[14,33] Each gelatin solution was placed in a 2.5 mL syringe with a stainless steel syringe needle (0.6 mm of inner diameter) connected to the anode of a power supply. The electrospun gelatin fibers were collected on aluminum foil covering the copper collector that was connected to the cathode of the power supply. All solutions were electrospun at controlled conditions of temperature (25 ± 2ºC) and an relative humidity (65 ± 5%).

Firstly, to study the effect of the type and concentration of gelatin on the electrospinnability and the diameter electrospun fibers, this procedure was followed: each gelatin was dissolved at different concentration (100, 120, 140, 160, 180 and 200 mg/ml) on the same solvent system formed by an aqueous solution of PBS(10X) and ethanol, at ratio 1:1 v/v. The resulting polymeric solutions were subsequently electrospun at the selected conditions of voltage, flow rate and distance between the needle and the collector that contributes to better electrospinnability. It is noteworthy to mention that for two different concentrations of gelatins (100 and 120 mg/ml) there was not any significant influence of operational parameters in the ranges of study: voltage (15, 18 and 21.5 kV), flow rate (0.75 and 1 ml/h), and distance between the needle and the collector at (9 cm).

Secondly, to evaluate the influence of the composition of the ternary solvent on the electrospinnability and fiber diameter, gelatin solutions of a fixed concentration (120 mg/ml) were prepared using two different series of solvent mixtures. On the one hand, solutions with three different PBS/ethanol ratios (3:2, 1:1, 2:3) maintaining the PBS concentration (10X) were tested. On the other hand, solutions with different PBS concentration (5X, 10X, 20X) were prepared maintaining the PBS/ethanol ratio (1:1).

2.3 Fiber characterization

The diameter and morphology of electrospun gelatin fibers were analyzed by Scanning Electron Microscopy (SEM) using a Phenom Standard SEM. Samples directly were observed, it is to say without any metallic coating. The average diameter of fibers was estimated by measuring the diameter of 50 arbitrary electrospun fibers using an image analyzing software package (ImageJ).

Besides, chemical structure of the electrospun fibers was analyzed by Fourier Transform Infrared Spectroscopy (FTIR) by using a Nicolet Avatar 320 spectrophotometer (Nicolet Instrument Corporation, USA). Samples were prepared by mixing 1 mg of fibers taken from the mat in a matrix of 300 mg of KBr followed by pressing (167 MPa). The spectrum was recorded in the range of 500 to 4000 cm^{-1}, averaging 32 scans at a resolution of 4 cm^{-1}.

Finally, the thermal properties of gelatin fibers were analyzed by Differential Scanning Calorimetry (DSC) by using a Perkin Elmer DSC7. During DSC measurements, a specimen (~ 4 mg) was heated from 50ºC to 300ºC at a heating ratio 20 ºC/min under a constant flow (50 ml(min) of nitrogen.

2.4 Cytotoxicity evaluation

Human foreskin fibroblasts (BJ-5ta) were used to determine the potential toxicity of the electrospun gelatin mats.

2.4.1. Cell culture

Cells were maintained in 4 parts Dulbecco's Modified Eagle's Medium (DMEM) containing 4 mM L-glutamine, 4500 mg/L glucose, 1500 mg/L sodium bicarbonate, 1 mM sodium pyruvate and 1 part of Medium 199, supplemented with 10 % (v/v) of fetal bovine serum (FBS), and 10 g/mL Hygromycin B at 37 ºC, in a humidified atmosphere with 5 % CO_2, according to the recommendations of the manufacturer.

The culture medium was replaced every 2 days. At pre-confluence, cells were harvested using trypsin-EDTA (ATCC-30-2101), 0.25 % (w/v) trypsin/0.53 mM EDTA solution in Hank's BSS without calcium or magnesium. Both BJ-5ta (ATCC-CRL-4001) and DMEM (ATCC-30-2002) were purchased from American Type Culture Collection (LGC Standards S.L.U, Spain).

2.4.2. Alamar Blue assay

Cultured cells were seeded at a density of 4.5 x 10^4 cells/well on 96-well tissue culture-treated polystyrene plates (Nunc) the day before experiments, exposed to dissolved gelatin mats (20 mg/mL in DMEM) at a final volume of 100 µL and incubated at 37 ºC in a humidified atmosphere with 5 % CO_2. Cells were examined after 24 h for signs of toxicity, using Alamar Blue assay. Resazurin, the active ingredient of AlamarBlue® reagent (Invitrogen, Life Technologies Corporation, Spain), is a non-toxic, cell-permeable compound that is blue in color and reduced to resorufin by viable cells, developing a red color compound.

After 24 h of contact with cells, the solution made of gelatin mats was removed, the cells washed twice with PBS and stained with AlamarBlue® reagent. 100 µL of 10 % (v/v) AlamarBlue® reagent in DMEM was added to the cells and incubated for 4 h at 37 ºC, after which the absorbance at 570 nm was measured, using 600 nm as a reference wavelength, in a microplate reader (Infinite M 200 plate reader, Tecan). The quantity of resorufin formed is directly proportional to the number of viable cells.
BJ5ta cells relative viability (%) was determined for each mat of electrospun fiber and compared with that of cells incubated only with cell culture medium. Hydrogen peroxide (500 µM) was used as a positive control for cell death.
All tests were performed by triplicate.

2.5. Statistical analysis

One-way analysis of variance (ANOVA) was used to determine the statistically significant differences between the average diameters of the electrospun fibers. The confidence interval was set at 95% and a p-value lower than 0.05 was considered to be a statistically significant difference. All statistical analyses were performed using Statgraphics Centurion XV.

3. RESULTS AND DISCUSSION

3.1. Effect of the operational parameters, type and gelatin concentration on the solutions electrospinnability

In previous works[14,22,34-36] it has been reported that the physicochemical properties of the dope solution (i.e. viscosity) are much more crucial for controlling the degree of electrospinnability and the final diameter of the electrospun fibers than the operational conditions (i.e. voltage or flow). Accordingly, for the present system, the observed differences should be mostly due to both the type and concentration of the electrospun gelatin or the nature and composition of the solvent.
To validate the aforementioned hypothesis, a preliminary study was carried out to evaluate the effect of voltage and flow rate on the fiber properties. The average diameter of electrospun gelatin nanofibers obtained at different operational conditions are reported in (**Table 1**) and results were analyzed by ANOVA **(Table 2)**. Taking into account the obtained results, it was provd that, for the studied ranges of operational parameters, only the type and concentration of gelatin had a significant effect on the electrospun fiber diameter ($F_{critical} < 0.05$). Thus, hereafter, all the solutions were electrospun selecting the optimal operational parameters (voltage and flow rate) that provided the best degree of electrospinnability in terms of the stability of the jet.
Besides, the SEM micrographs shown in **Figure 1** display the morphology of the electrospun mats obtained from the three types of gelatin at different concentrations using a single solvent composition, namely PBS(10X)/Ethanol, 1:1 v/v. As it can easily observed, some differences on morphology occurred and were associated to both the gelatin concentration and its type, as it was initially suggested. For instance, nanofibers with beads were more often found in those mats prepared with a low concentration of gelatin BS, which had the lowest Bloom.

Table 1. Average diameter of gelatin fibers obtained at different operational conditions of voltage (15, 18, 21.5 V) and flow rate (0.75, 1 ml/h). Three types of gelatins (BS, BB, PS) and two concentrations (100 and 120 mg/ml) were tested**.** Uncertainty corresponds to the standard deviation of 50 measures.

Flow Rate (ml/h)	Voltage (kV)	Type of Gelatin	[Gelatin] (mg/ml)	Average diameter (nm)
0.75	18	BS	100	97 ± 12
0.75	21.5	BS	100	102 ± 14
0.75	15	BS	100	105 ± 14
0.75	15	BS	120	130 ± 15
0.75	18	BS	120	127 ± 13
0.75	21.5	BS	120	123 ± 12
0.75	21.5	BB	100	168 ± 17
0.75	18	BB	100	164 ± 15
0.75	15	BB	100	164 ± 17
0.75	18	BB	120	186 ± 21
1.0	15	BB	120	194 ± 18
0.75	15	BB	120	206 ± 21
075	15	PS	100	304 ± 37
1.0	15	PS	100	316 ± 43
1.0	18	PS	100	319 ± 38
1.0	18	PS	120	429 ± 68
1.0	15	PS	120	418 ± 79
0.75	15	PS	120	413 ± 72

Table 2. ANOVA analysis to assess the effect of voltage, flow rate and type and concentration of gelatin on the diameter of electrospun fibers.

Source of variance	Sum of Squares	Degrees of freedom	Mean square	F	Critical value of F (p=0.05)
Flow Rate	2014.95	2	1007.48	2.09	0.1741
Voltage	52.3409	2	26.1705	0.05	0.9474
Gelatin Type	96119.2	2	48059.6	99.83	0.0000
Concentration	9863.57	1	9863.57	20.49	0.0011
Error	4814.23	10	481.423		
Total	225274.	17			

The reason is that at low concentrations of polymer the viscosity of the solution was also low (see **Figure 2**) and the high surface tension of polymer solutions leaded to the instability and the breakup of the solution jet into droplets.[37] It is worth to mention that the incidence of beads decreased either when increasing the concentration or when increasing the Bloom of the gelatin. Both factors (concentration and Bloom) improved the quality of fibers because the electrospining jet was stabilized due to the increase of the solution viscosity that, in turn, was related to the intensification of polymer chain entanglement. As a result, bead-free nanofibers were obtained for BS gelatin at 200 mg/ml and for BB and PS gelatins at any of the tested gelatin concentrations.

However, when viscosity was too high the solutions of gelatin were found unspinnable. This was the case for solutions of the gelatin with the highest Bloom (PS) at concentration ≥ 180 mg/ml.

Figure 2 depicts the evolution of dynamic viscosity versus gelatin concentration for the three different tested gelatins. As it has been reported previously, viscosity follows an allometric ($\eta = \alpha c^{ß}$) relationship with the concentration of gelatin.[15,22,34,38] More concretely, the relationship between dynamic viscosity and concentration of gelatin in semidilute regime is usually given by the equation proposed by De Gennes (**Equation 1**):

$$\frac{\eta}{\eta_s} = \eta_r = \left(\frac{c}{c^*}\right)^{3/(3\nu-1)} \qquad \textbf{(Equation 1)}$$

where η_r is the relative viscosity, η_s is the viscosity of the solvent, c* is the critical chain overlap concentration and ν is the Flory exponent, which has been widely used to characterize polymer-solvent intermolecular interaction giving yield to the so-called good, theta and poor solvent behavior.[39]

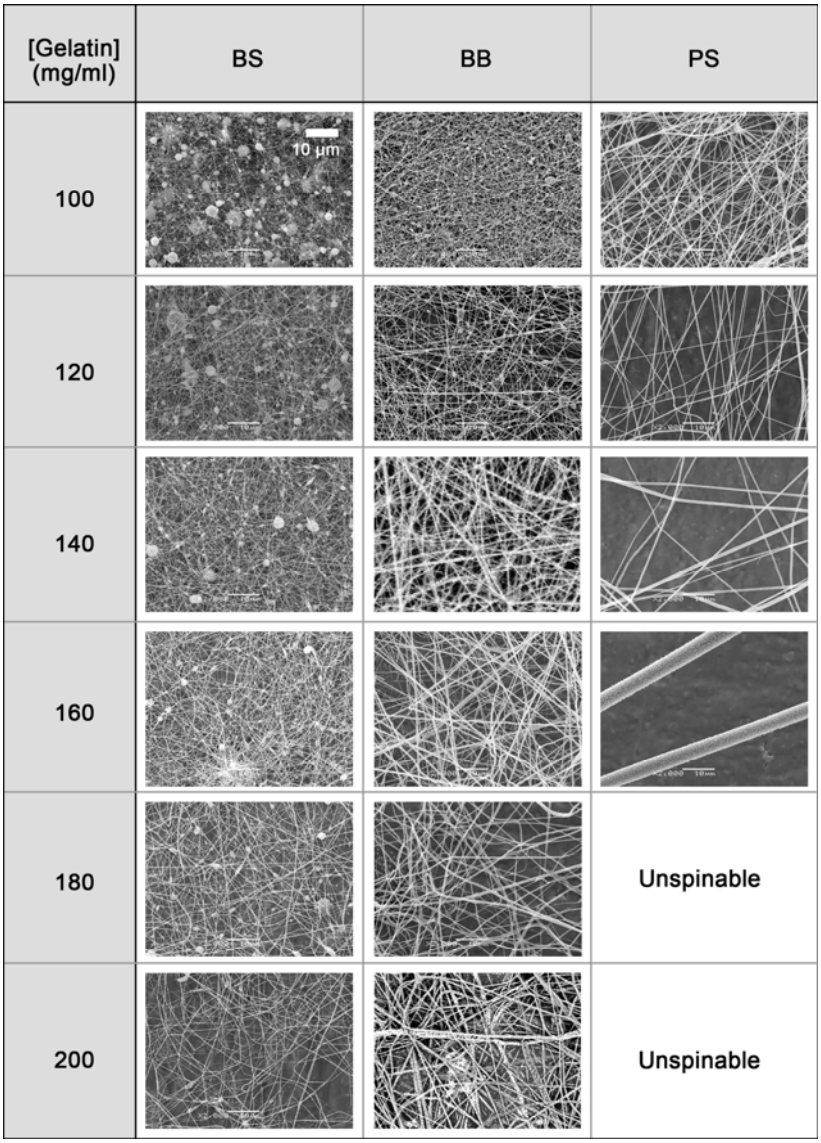

Figure 1. SEM images of electrospun fibers obtained from BS, BB and PS gelatin solutions at different concentration showing morphology and electrospinnability. PBS (10X)/ethanol ratio 1:1 v/v.

In fact, the parameter c* marks the onset of significant polymer chain overlap in solution and can be estimated by using **Equation 2**[40]:

$$c^* = \frac{2.5}{[\eta]} \qquad \textbf{(Equation 2)}$$

where $[\eta]$ is the intrinsic viscosity, defined as the increase in viscosity of a solvent through the addition of an infinitesimal amount of solute. In our case, $[\eta]$ was experimentally determined by measuring the dynamic viscosity of diluted solutions and extrapolating to

infinite dilution by the well-known Huggins equation (**Equation 3**), where η_{sp} is the specific viscosity of the polymer solution and K_H is the Huggins constant.[41-42]

$$\frac{\eta_{sp}}{c} = [\eta] + K_H[\eta]^2 c \quad \textbf{(Equation 3)}$$

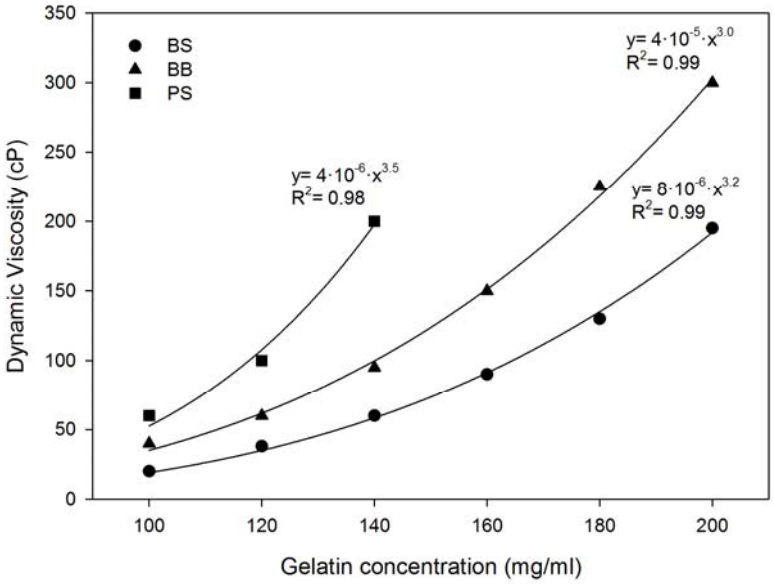

Figure 2: Dynamic viscosity of solutions vs. gelatin concentration for each gelatin type.

The intrinsic viscosities of the different gelatins, [η], estimated for the solutions using PBS(10X)/ethanol 1:1 v/v as a solvent, are reported in **Table 3**. Based on the estimated values of [η], the c* concentration was then theoretically calculated for each system by using **Equation 2** and the corresponding values are also reported in the same table. It is worth to note that c* was lower for gelatins with higher Bloom ($c^*_{PS} < c^*_{BB} < c^*_{BS}$) indicating that in this case chain overlapping occurred at lower concentration of gelatin compared to solutions prepared using gelatin of lower Bloom.

Table 3. Intrinsic viscosities, [η] of the tested gelatins calculated by Huggin's equation and the calculated critical chain concentration (c*).

Gelatin type	Bloom (g)	[η] (ml/mg)	c* (g/ml)
BS	225	0.29	8.6
BB	250	0.33	7.5
PS	300	0.43	5.8

Following De Gennes' equation (**Equation 1**) the relationship between relative viscosity and c/c* ratio allows to establish the regime of work (dilute, semidilute untentangled or semidilute entangled) based on the changes in the slope of the plot of **Figure 3**.[43] In this case, taking into account that c/c* > 1 for all the experiments and that these data fitted well to a single straight line of slope 3.11, it can be concluded that all the experiments were carried out on a semidilute entangled regime, where the gelatin concentration was high enough to induce a significant degree of entanglement between polymer chains. As it has been said before, the scaling exponent is directly related to the Flory exponent (**Equation 1**). Here, the estimated Flory exponent was 0.65 indicating that the solvent (PBS(10X)/Ethanol 1:1 v/v) behaves mostly as a good solvent for the three tested

gelatins (the theoretical value for a good solvent is 0.6). Note that the same behavior has been corroborated for alternative systems such as gelatin/acetic acid solutions.[14]

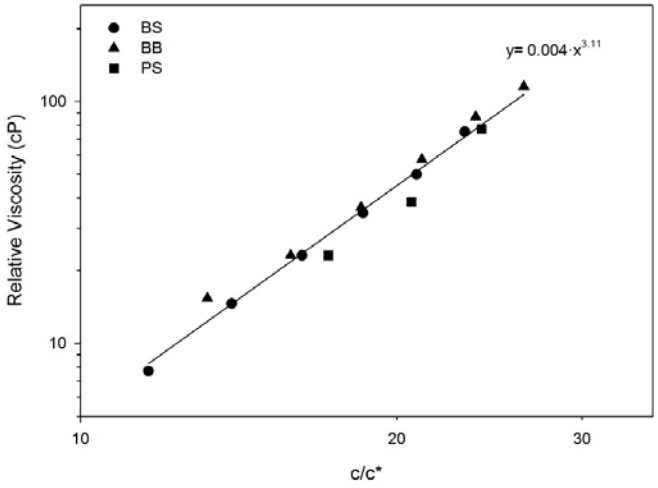

Figure 3: Relative viscosity versus c/c* of the three gelatins.

3.2. Effect of the type and gelatin concentration on the fiber diameter.

After having seen how the type and concentration of the gelatin solution determined the viscosity of the dope solution and, as a result, influenced the electrospinnability and morphology of gelatin mats, a comprehensive study of the effect of the viscosity on the diameter of the electrospun fibers was carried out. The influence of the gelatin concentration on the diameter of the electrospun fibers is depicted in **Figure 4**. Results indicated that an increase of the average diameter of the nanofibers was produced with the increasing of gelatin concentration. This behavior followed the same trend regardless of the type of gelatin although with different intensity and was in agreement with the results obtained by Zha et al. based on the electrospinning of porcine skin gelatin.[31]

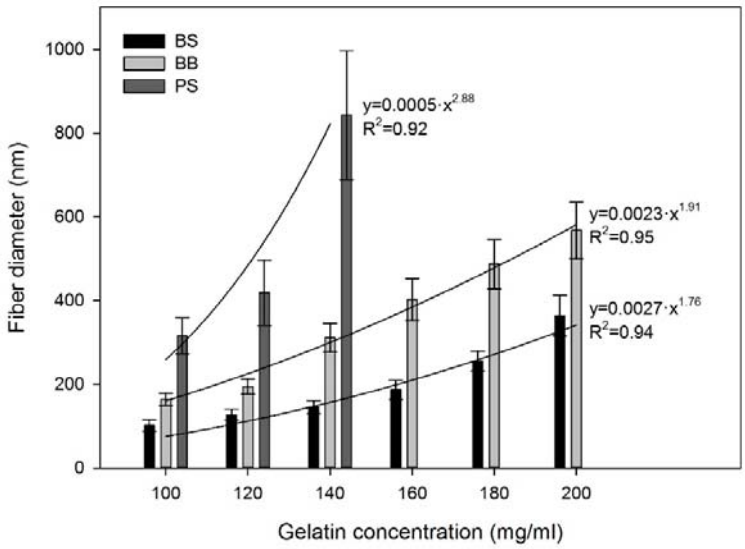

Figure 4. Average diameter of the electrospun gelatin fibers obtained from BS, BB and PS gelatins at different concentration.

As regard to the relationship between the diameter of the electrospun fibers and the concentration of the gelatin solutions, the following allometric equation is often used to correlate the data[34,44-46]:

$$d = \alpha(Be)^{\beta}$$ (**Equation 4**)

where Be is the so-called Berry number (Be), a dimensionless number defined as the product of the polymer concentration (c) and the solution intrinsic viscosity (Be=c*[η]) and it accounts for the regime of electrospinnability and the diameter of the resulting fibers.

When the average diameter of the fibers was plotted against the Be number in **Figure 5** (both axes at logarithmic scale, a linear relationship was found for each types of gelatin although the scaling exponent β varied from one polymer to another (1.76,1.91 or 2.9 for PS, BB or BS, respectively). Thus, it was not possible to obtain a unique relationship between the diameter and the dimensionless number Be that encompassed the behaviour of the three solvent-gelatin systems. This fact is quite coherent since each of the tested polymers bears a different chain length and, therefore, a different molecular weight and dissimilar rheological properties.

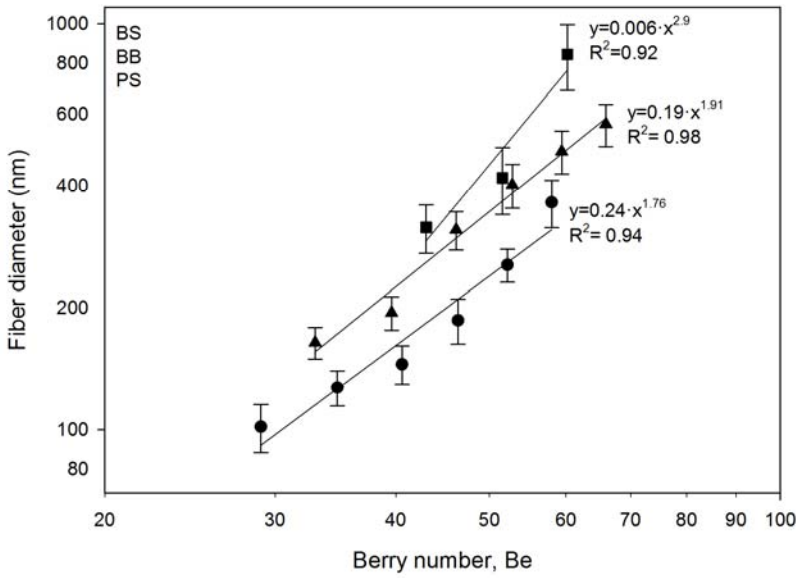

Figure 5: Average diameter of electrospun fiber versus Berry number for the three tested gelatins.

However, from a practical point of view, it would be advantageous to find a simple way to consider the three gelatins as if they were a single system so that knowing Be (or a similar parameter) one could predict the average diameter of the obtained fibers in a quite accurate manner. Empirically, among several possible approaches, it was observed that the product Be·[η] provided a single and acceptable fitting (R^2 = 0.94) for the data of the three tested gelatins, thus unifying the behaviour of the three systems (see **Equation 5** and **Figure 6**).

$$d = \alpha(Be[\eta])^{\beta} \quad \textbf{(Equation 5)}$$

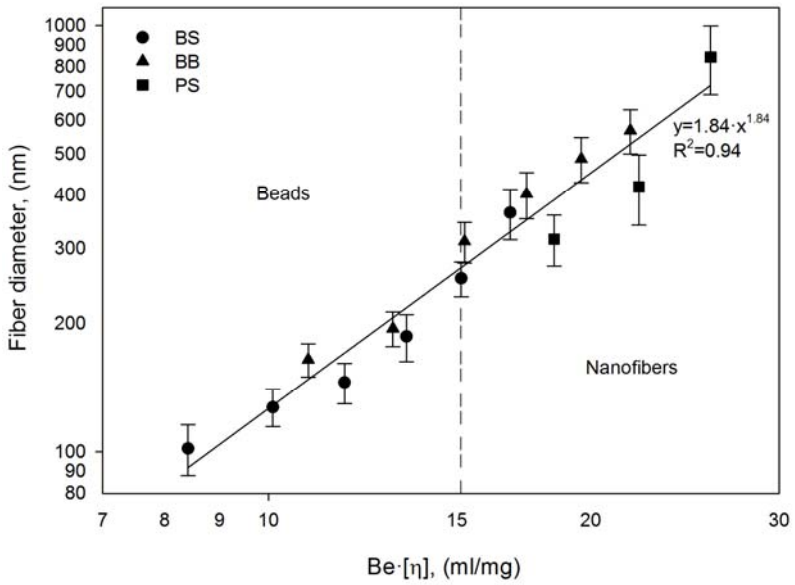

Figure 6: Average diameter of electrospun fiber versus Be·[η] for the three tested gelatins.

Taking into account that studied proteins mainly differ from the molecular weight which strongly influences the value of [η]; it seems consistent that the correction of Be was made with [η].

Moreover, and thanks to this representation, it is feasible to clearly identify two different electrospinability domains: nanofibers for Be·[η] > 15 and beaded-fibers for Be·[η] < 15. Therefore, this kind of representation results in a useful tool for predicting not only the nanofiber diameter but also their morphology.

3.3. Effect of the solvent composition

As it has been aforesaid, solvent properties have an important role in the electrospinning of gelatin at room temperature since the medium determines the polymer-solvent intermolecular interactions.[4] In this regard, any change in the composition of the water/PBS/ethanol ternary mixture would directly affect the physicochemical properties of the dope solution. For that reason, the effect of the composition of the solvent upon the electrospinnability of gelatin and fiber diameter was studied.

Firstly, the influence of ethanol content was studied by electrospinning some solutions prepared with different amount of ethanol and PBS(10X) and containing any of the three studied gelatins at a fixed polymer concentration (120 mg/ml). A this point it is important to state that gelatin is not soluble on pure ethanol, so a minimal amount of PBS might be necessary to prepare the solutions, whereas high concentrations of PBS do induce the gelation process (what would hinder the electrospinnig process). Consequently, a noticeable but not excessive amount of ethanol is indeed required to break down hydrogen bonding between gelatin and water.[47] Considering this, three PBS(10X)/ethanol volume ratios (3:2, 1:1 and 2:3) were chosen to prepare the tested solutions of gelatin. Note that, within the selected ranges of composition, which were in agreement with previous works[31], homogeneous solutions were produced for each gelatin type.

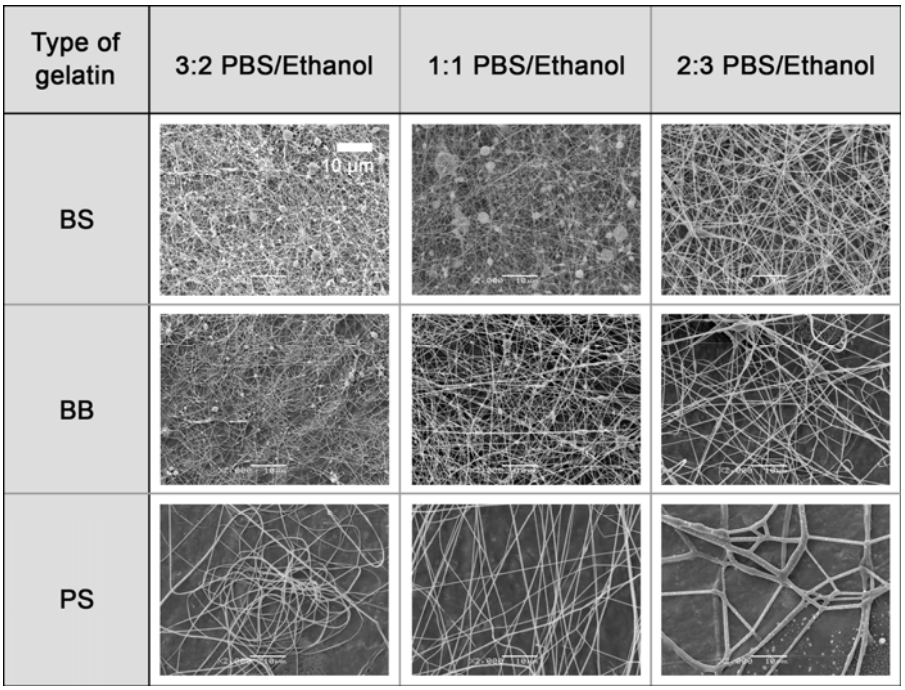

Figure 7: SEM micrographs of nanofibers mats from different gelatin type and ratio PBS/ethanol.

The morphology of the mats and the average diameter of the resulted nanofibers are illustrated in **Figure 7** and **Figure 8**, respectively. From the results it can be seen that gelatins showed a similar behavior when the ratio PBS/ethanol decreased: free-beads nanofibers mats with fiber of higher diameter were obtained when electrospinning solutions with the lowest PBS/ethanol ratio. According to the literature[48] , the observed trend is due to the decrease of conductivity of the dope solution that occurs when increasing the amount of ethanol or decreasing the amount of salts coming from PBS solution. Both effects are maximized for the 2:3 PBS/ethanol medium. As a result of the decrease of conductivity, the solution jet is less stretched under a high electric field increasing the diameter of the electrospun fibers.

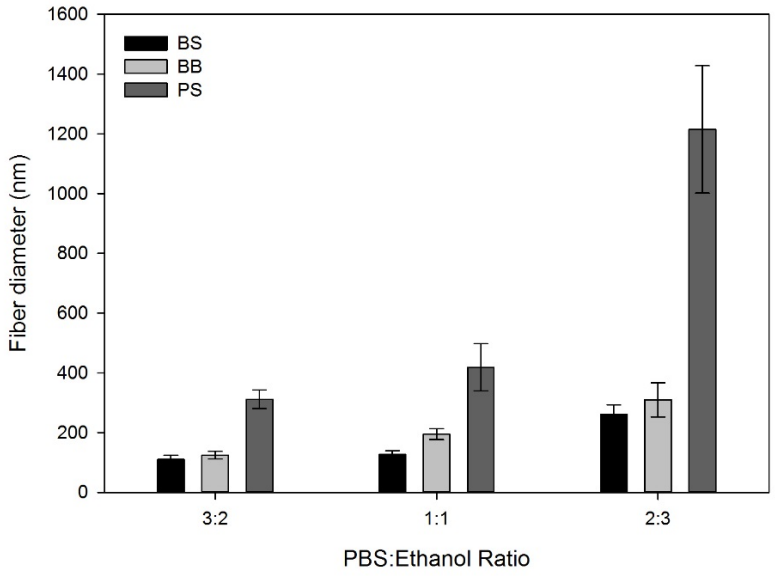

Figure 8. Average fiber diameter as a function of PBS(10X)/ethanol ratio

Secondly, the influence of the ionic strength of the solvent on the electrospinnability of gelatin solutions and on the morphology of the obtained nanofibrous mats was analyzed in detail. For that purpose, gelatin solutions at a concentration of 120 mg/ml were prepared in 1:1 PBS/Ethanol solvent using solutions of PBS of different concentration (5X, 10X and 20X). In this case, the total amount of ethanol was the same for all the studied samples, as well as the gelatin/ethanol ratio. The studied concentrations of the PBS solutions were chosen taking into account that gelatins are not soluble on pure ethanol nor in 1:1 water/ethanol mixtures[31] and therefore a minimal amount of ions (Na^+ and K^+) must be added to break down the gelatin networks and form the solution.
Results regarding the morphology of the nanofibers mats and their average fiber diameter can be seen in **Figure 9** and **Figure 10**, respectively. In this case, those solutions containing low salt concentration (<10X) formed uneven fiber mats with large amount of beads, where, in addition, the salt crystals were very noticeable. Increasing the ionic strength the uniformity of the mats increased and, at the same time, fiber diameter decreased (**Figure 10**), along with the presence of the crystals. These results are in agreement with the previous literature[31] and are also related with the change of the solution conductivity: an increase of the ionic strength enhances the solution conductivity and, consequently, the solution jet is stretched under high electric voltage, which lead to fabricate a smaller fibers and more uniform fiber mats.

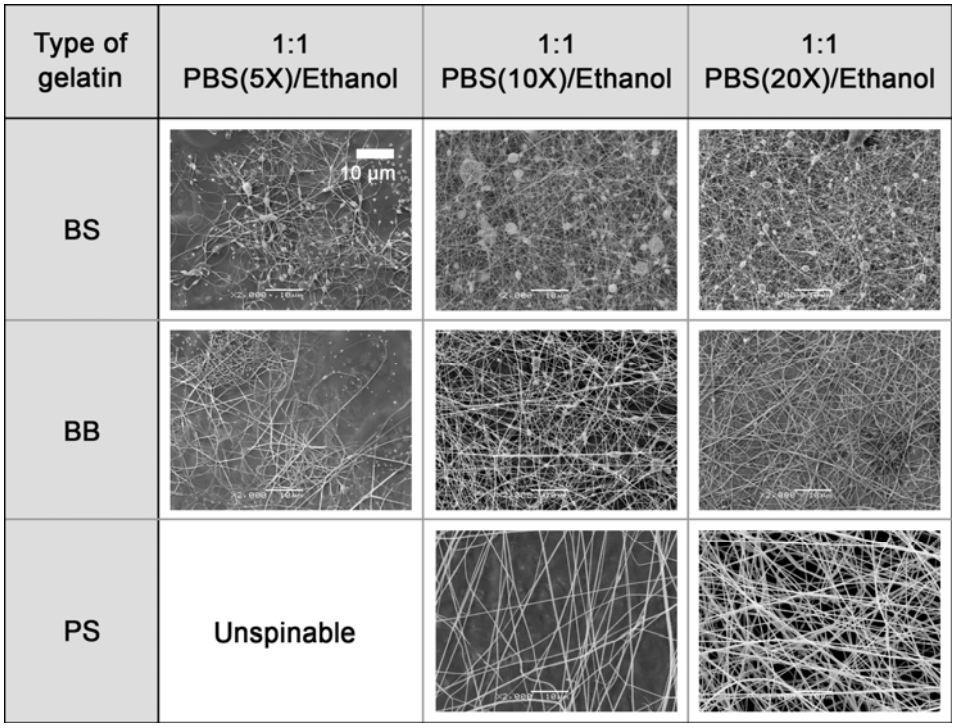

Figure 9: SEM micrographs of nanofibers mats of the three types of gelatins obtained varying the PBS concentration.

Although the behavior of both morphology and diameter of electrospun fibers as a function of PBS concentration is similar for the three gelatins, it is important to note that the amount of salt necessary to obtain a homogeneous solution (for the same gelatin concentration) is different depending on the gelatin and it is directly related to the gelatin gel strength. For this reason, solutions of PS gelatin in 5X PBS were not suitable for electrospinning (**Figure 9**).

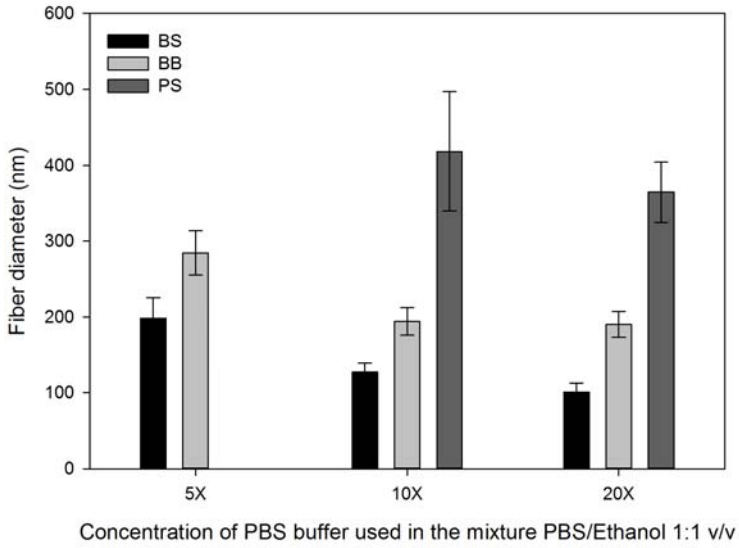

Figure 10: Average fiber diameter as a function of PBS solution concentration.

3.4. Nanofibers characterization

So as to compare the chemical structure of electrospun gelatins and their original counterparts in powder form, both kinds were analyzed by FTIR. Specimens of nanofibers mats with a similar average diameter were selected to avoid any differences related to fiber solvent sorption on the analysis. Hence, the mats samples were prepared by electrospinning solutions of 200 mg/ml for BS, 140 mg/ml for BB and 100 mg/ml for PS gelatin. The solvent composition and ionic strength was the same for all three samples (PBS (10X)/Ethanol, 1:1). The results are shown in **Figure 11** and, despite the fact that the spectra of powder gelatins were much more attenuated and smooth, all the spectra showed the characteristic IR bands of gelatin at ~3300, ~1650, ~1540 and ~1240 cm^{-1} corresponding to the Amide A (N-H stretching vibration), Amide I (C=O stretch), Amide II (N-H bend and C-N stretch) and Amide III (C-N stretch plus and N-H deformation), respectively.[49]

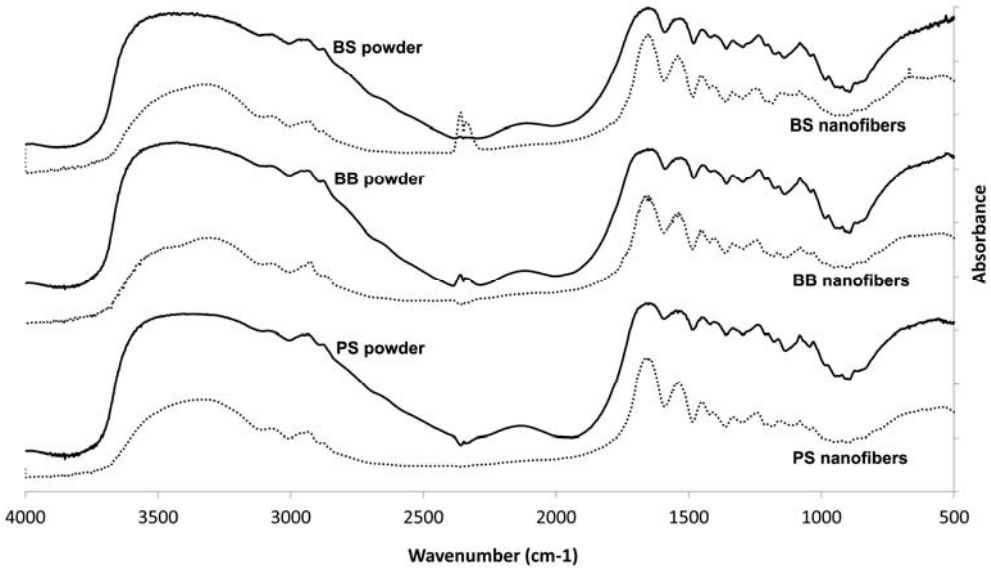

Figure 11: FTIR spectra of different gelatin type solutions dissolved in PBS(10X)/ethanol 1:1 v/v before (powder) and after (mat) the electrospinning process.

From these results it could be concluded that no change in the chemical structure of the gelatin occurred during the electrospinning process, as it had also been reported in many cases.[22,49] However, the FTIR technique might not be sensitive enough to underline small structural differences between samples, particularly those related to protein conformation. Therefore, Differential Scanning Calorimetry (DSC) analyses were carried out to check whether any denaturalization process affected the chemical structure of three different gelatins either at the dissolution step or at the electrospinning process. The DSC thermograms of the three studied gelatins, both before and after their dissolution on the ternary mixture based solvent (PBS(10X)/ethanol, 1:1v/v), are shown in **Figure 12**.

Conversely to what happened by FTIR analysis, strong differences between pure gelatins and their analogue mats of fibers were observed at the characteristic peaks corresponding to the helix to coil transition temperature (T_g, first peak in the range 90-110ºC) and to the degradation point (T_m, second peak in the range 200-230ºC).

Both T_g and T_m were significantly lower for all the mats ($T_{g,n}$ = 95 ºC and $T_{m,n}$ = 200 ºC) compared to the thermal properties of the powder gelatins ($T_{g,p}$ = 110 ºC and $T_{m,p}$ = 230 ºC). In fact, for the nanofibers, the T_m was only partially identified as a shoulder in the thermograms. According with the literature[20-52], the dissolution step or/and the electrospinning process negatively affect the gelatin thermal stability due to the destabilization of its original chemical structure. Based on previous results carried out with acetic acid as a solvent[27] that confirm that electrospinning process is not the main issue affecting the chemical structure of the gelatin fibers, it can be concluded that dissolution step of gelatin is the responsible of the loss of thermal properties of gelatin.

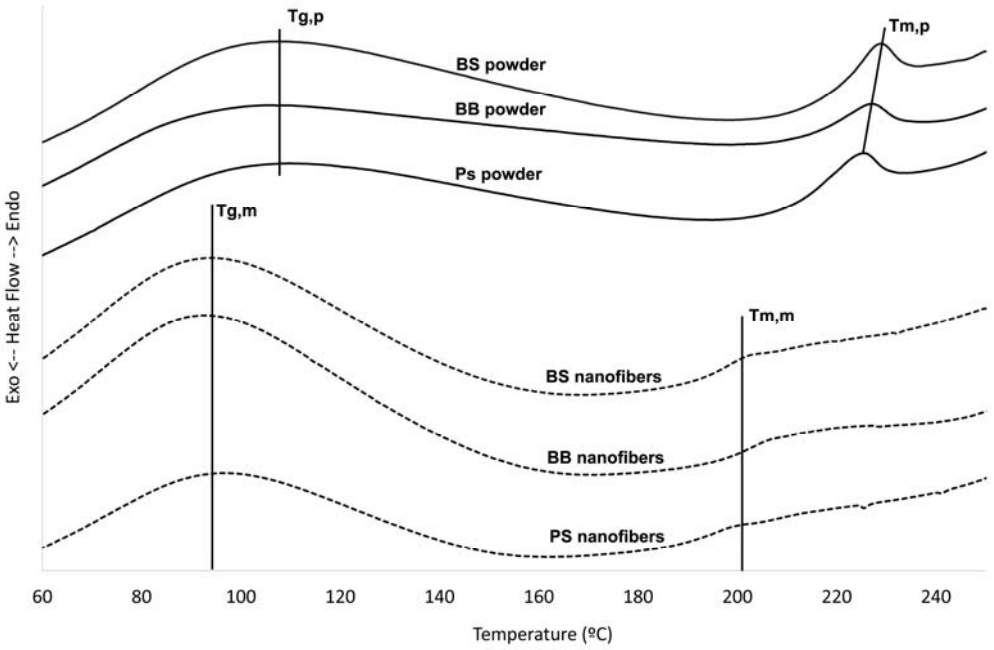

Figure 12: Differential Scanning Calorimetry (DSC) thermograms of the three different gelatins dissolved in PBS(10X)/ethanol 1:1 v/v solvent before (powder) and after (mat) the electrospinning process.

Nonetheless, it is worth to note that the decomposition peaks of the original gelatins agreed with the ones published in the literature[22,25]: ~ 230ºC for BS gelatin, ~ 228 ºC for BB and ~ 225 for PS, indicating an indirect relationship between the gelatin Bloom and the decomposition temperature.

3.5. Cytotoxicity evaluation

To assess cytotoxicity of the nanofibers mats, which may be caused by the presence of traces of solvent in the electrospun fibers, the Alamar Blue cell viability assay[53] was carried out using BJ-5ta fibroblasts cells. First, the nanofibers mats were dissolved in the culture medium and then put in contact with the cell during an incubation period. Results of Alamar blue assay are shown in **Figure 13** and demonstrated that the mats of gelatin perform well in relation to cell viability with values higher than 90% regardless the type of gelatin. Consequently, it is possible to affirm that PBS/ethanol solvent mixture was not negatively affecting cell viability. These results were comparable those recently obtained for a similar system which used acetic acid aqueous solutions as alternative solvent where a cell viability of 90% was achieved.[27]

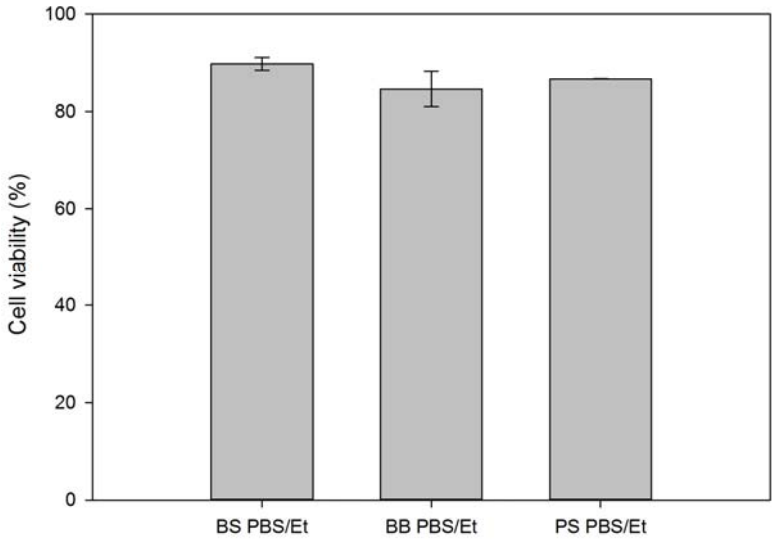

Figure 13: Cell viability of BJ-5ta fibroblast cells as a function of gelatin type.

4. CONCLUSIONS

Different compositions of PBS/Ethanol solvent mixture were investigated so as to determine their feasibility to dissolve and electrospin three different gelatins at room temperature. It was demonstrated that the size of electrospun nanofibers decreases due to the high stretching of the solution jet when increasing the ionic strength or the PBS/ethanol ratio.

In addition, an increase of the gelatin concentration or the gelatin Bloom induces an increase of the solution viscosity and, consequently, the average fiber diameter increases.

Besides, the calculation of intrinsic viscosity for each gelatin type in the PBS(10X)/ethanol 1:1v/v solvent allowed us to determine that the solvent mixture acts as a good solvent for all the tested gelatins. The allometric relationship between the Berry number and the diameter of the electrospun fiber is demonstrated for each individual gelatin and, in addition, thanks to a simple modification of the Berry number (namely the use of $Be \cdot [\eta]$) a new allometric relationship was found. This relationship is able to take into account the effect of the different types of gelatin and provides an easy way to predict the fiber diameter for any of the tested gelatins.

When evaluating the physicochemical properties of the obtained nanofibers, it was proved that gelatins are affected by either the dissolution (most probable) or the electrospinning processes since a change on the thermal properties was noticed by DSC

with a slight decrease of both helix to coil transition temperature (T_g) and degradation point (T_m) of the electrospun mats compared with the pure gelatins.
Finally, gelatin mats fabricated with PBS/ethanol solvent mixture perform well in relation to cytotoxicity as all the fabricated mats obtained cell viability values above 90%, regardless the gelatin type.

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