Electrospinning of gelatin fibers using solutions with low acetic acid concentration: effect of solvent composition on both diameter of electrospun fibers and cytotoxicity

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ABSTRACT

Gelatin fibers were prepared by electrospinning of gelatin/acetic acid/water ternary mixtures with the aim of studying the feasibility of fabricating gelatin nanofiber mats at room temperature using an alternative benign solvent by significantly reducing the acetic acid concentration. The results showed that gelatin nanofibers can be optimally electrospun with low acetic acid concentration (25% v/v) combined with gelatin concentrations higher than 300 mg/ml. Both gelatin solutions and electrospun gelatin mats (prepared with different acetic acid aqueous solutions) were analyzed by FTIR and DSC techniques in order to determine the chemical and structure changes of the polymer. The electrospun gelatin mats fabricated from solutions with low acetic acid content showed some advantages as the maintenance of the decomposition temperature of the pure gelatin (~230ºC) and the reduction of the acid content on electrospun mats, which allowed to reach a cell viability upper than 90% (analyzed by cell viability test using human dermal fibroblast and embryonic kidney cells). This study has also analyzed the influence of gelatin and acetic acid concentration both on the solution viscosity and the electrospun fiber diameter, obtaining a clear relationship between these parameters.

Keywords: electrospinning, nanofibers, mat, cell viability, gelatin, acetic acid.

1. INTRODUCTION

In recent years, numerous reports in the field of tissue engineering have put the emphasis on the design and manufacturing of biocompatible and biodegradable supports with capacity of mimicking the structural and functional properties of extracellular matrices (ECM).[1-3] For an optimal biocompatibility, scaffolds used for tissue engineering should possess special characteristics of degradation, porosity, microstructure, size, etc.[1,3] These characteristics highly depend on the fabrication method and, consequently, different techniques for the production of such scaffolds have been investigated and optimized (e.g. self-assembly, phase separation).[1,2,4] More recently, the electrospinning technique appeared as a versatile technique for manufacturing nanofibers and nanofibrous arrays with dimensions and scale similar to those of the native ECM[5-11], suitable for medical applications.[12]

The electrospinning technique[11] allows the production of small diameter fibers (ranging from nanometers to micrometers) by applying a high voltage electrostatic field between a metal capillary syringe containing a polymer solution and a grounded collector where the fibers are deposited. During this process, as a result of solvent evaporation, electrospun fibers are deposited onto the collector in the form of nonwoven fibrous webs of high porosity. The properties of the obtained fibers depend on the operating conditions, e.g. flow rate, voltage, time, temperature and distance from the collector, as well as on the properties of the polymer solution, e.g. concentration, density, viscosity, conductivity, surface tension.[7,13]

Electrospinning can be applied to both synthetic[14-16] and natural polymers, including polysaccharides[17] and proteins, being collagen[18,19], silk fibroin[20] and gelatin[21] the most studied ones. Gelatin is known to have biocompatibility and biodegradability similar to collagen.[22,23] In fact, it is easily obtained by partial hydrolysis of collagen from animal tissues such as skin, muscle, and bone. Depending on the hydrolysis method, two different types of gelatin are produced: type A gelatin (acid process), type B (alkaline process). Both gelatins differ mainly in their amino acid composition, polypeptides pattern, bloom strength, turbidity and foaming properties.[24,25]

From a technical point of view, the most important parameter influencing the electrospinning manufacturing process of gelatin nanofibers is the solvent selection[26] because, although gelatin is soluble in warm water, the electrospinning cannot be done at room temperature due to the gelation process that occurs between gelatin and water, which increase the solution viscosity avoiding the flow of aqueous gelatin solutions into the syringe.[27]

With the aim of avoiding the gelation process and allow the electrospinnability of gelatin solutions, some complex solvent such as 1,1,1,3,3,3-hexafluoro-2-propanol (HIPF) or 2,2,2-trifluoroethanol (TFE) have been proposed for the fabrication of scaffolds made of natural polymer such as collagen.[21,28-32] However, due to their highly corrosive nature, these solvents may affect the original protein structure[11,33,34] besides providing a potential cytotoxicity to the obtained scaffolds, since the presence of small amounts of residual solvent embedded on the electrospun fibers is almost unavoidable.[35,36] This fact, combined with the high cost of these solvents, promoted the search of alternative systems to electrospin gelatin such as: i) the use of gelatin aqueous solution at high temperatures[37,38], ii) blending gelatin with another biopolymer (e.g. sodium alginate[38], poly(ethylene oxide), poly(e-caprolactone))[11], PLA[39], iii) using solvent mixtures (acetic and ethyl acetate[40], and iiii) and using of carboxylic acid (formic acid[22,41] or acetic acid[26,39-43]).

Among these alternatives, the use of aqueous solutions of carboxylic acids has been postulated recently as a preferable option to dissolve and electrospin gelatin at room temperature. The use of these acids implies a clear advantage over HIPF and TFE solvents due to their lower cytotoxicity and their simplicity of processing compared to other alternatives. However, the concentrations of acid proposed up to now to electrospin gelatin are quite high (minimum of 60% v/v), inducing the partial decomposition of gelatin and adversely affecting the structural integrity of the nanofibers.[22]

This study analyzes the feasibility to electrospin nanofiber mats of gelatin at room temperature using an acetic acid based solvent characterized by a low concentration of acid. The effect of the acidity of the solvent on both the gelatin structure and the cytotoxicity of the final mat were tested. Finally, the relationship between reagents concentration, solution viscosity and fiber diameter was studied given that the

diameter of the fibers is a crucial parameter for instance, to mimic the size of the fibers composing the extracellular matrix of connective tissue.

2. EXPERIMENTAL

2.1. Materials

Gelatin powder from bovine skin (type B with bloom ~225 g) was purchased from Sigma Aldrich (Spain) and used without further treatment or purification. Glacial acetic acid (99.99%, Panreac, Spain) and bi-distilled water were used as solvents. Solutions of gelatin with concentrations 200, 250, 300, 350, 400 mg/ml (kg/m^3) were prepared using acetic acid or mixtures of acid-water (25%, 50%, 75% v/v of acetic acid) as solvents. Gelatin was dissolved at room temperature (23 ºC) by stirring for 1 hour.

2.2. Electrospinning process

Electrospinning was performed in a home-engineered device.[44,45] Each gelatin solution was loaded into a 2.5 ml syringe with a stainless steel syringe needle (0.6 mm inner diameter) connected as an anode to a high voltage power supply. About 6-10 cm below the needle, a flat copper collector (connected as a cathode to the power supply) was placed to receive the electrospun fibers. The flow rate was controlled by a pump, and set between 1-1.5 ml/h, depending on the solution requirements. The applied voltage was in the range of 15-18 kV and all solutions were electrospun at room temperature (23 ºC).

Electrospun mats were not chemically cross-linked for mechanical stabilization to avoid interferences during their chemical and structural characterization.

2.3. Viscosity measurements

The viscosity of the different solutions was determined using a viscometer (Brookfield DV-II +, USA). After mixing for 1 h, samples were stored for different times (0 h, 1 h, 3 h, 24 h) before the measure of viscosity, in order to follow the gelation process. Each solution viscosity was measured three times and results shown a standard deviation below 2%.

2.4. Electrospun fibers characterization

The diameter and distribution of the electrospun gelatin fibers were directly examined by Scanning Electron Microscopy (SEM) (Phenom Standard, Phenom-World, Netherlands) without any metal coating. The obtained images were processed by image analyzing software (Photoshop CS6, Adobe, Ireland) so as to determine the average diameter and its standard deviation. Typically, 50 arbitrary fibers were measured.

Besides, both chemical structure and conformation of as-made fibers were 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 fiber mat in a matrix of 300 mg of KBr followed by pressing (167 MPa). The spectrum was recorded in the range of 400 to 4000 cm^{-1} and averaging 32 scans at a resolution of 4 cm^{-1}.

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

2.5. Cytotoxicity evaluation

2.5.1. Cell culture

To determine the potential toxicity of the mats of gelatin fibers obtained from solutions with different acetic acid content, human foreskin fibroblasts (BJ-5ta) and Human Embryonic Kidney cells (HEK 293T) were used. Cells were maintained in 4 parts of 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) whereas HEK 293T was purchased from the European Collection of Cell Culture.

2.5.2. Alamar Blue assay

Cells were seeded at a density of 4.5 x 10^4 cells/well on 96-well tissue culture-treated polystyrene plate (Nunc, Thermo Scientific, USA) the day before experiments. Then, they were exposed by indirect contact to the electrospun gelatin fibers, previously dissolved on medium (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 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 it can be reduced to resorufin by viable cells, developing a red color compound. After 24 h contact with cells, the solution of dissolved fiber mats was removed, the cells washed twice with PBS and stained with AlamarBlue® reagent. 104000 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, USA). The quantity of resorufin formed is directly proportional to the number of viable cells. BJ5ta cells relative viability (%) was determined for each concentration of acetic acid and compared with that of cells incubated only with cell culture medium (negative control, C-) whereas H_2O_2 500 µM was used as a positive control (C+) of cell death. All tests were performed in triplicate.

2.5.3. Cells morphology

Morphological changes in cells were also followed by phase contrast microscopy using an Eclipse Ti-S microscope (Nikon Instruments Inc., Netherlands), after 24 h of contact with gelatin mats.

3. RESULTS AND DISCUSSION

3.1. Viscosity of solutions

Regarding the electrospinning process, besides some technical parameters such as voltage, distance and flow rate, there are several other important parameters that influence the electrospinnability of solutions such as surface tension, conductivity, viscosity and molecular weight.[11] For instance, surface tension determines the upper and lower boundaries of electrospinning window if all other variables are held constant.[46] Previous studies concluded that the increase of an acid concentration provokes a surface tension decrease[40] that benefits the electrospinnability, normally impeded by high surface tensions.

In this case, to study the effect of viscosity on the electrospinnability, firstly, the viscosity changes over the storage time were analyzed for several gelatin solutions prepared with different solvent mixtures (25 - 100% v/v acetic acid). As shown in **Figure 1**, the viscosities of those solutions prepared with aqueous acetic acid at 50% and 75% and pure acetic acid (100%) vary depending on gelatin concentration (200 - 400 mg/ml) but they were very stable with time since no significant variation could be observed up to 24 h after mixing (the maximum increment of 15% was attained for the highest gelatin concentration solution, 400mg/ml, in 100% acetic acid), contrarily that occurs for pure formic acid system, where the viscosity of gelatin hardly decreases after 5 hours of storage time.[22] In contrast, for the gelatin solutions containing 25% acetic acid, the viscosity clearly increased with time being the most important increment observed for the most concentrated solution in terms of gelatin content (about a 300% of increment). These changes of viscosity make sense taking into account the gelation phenomenon that gelatin undergoes in the presence of a high amount of water[47], which is also proportional to the gelatin concentration in solution.[48]

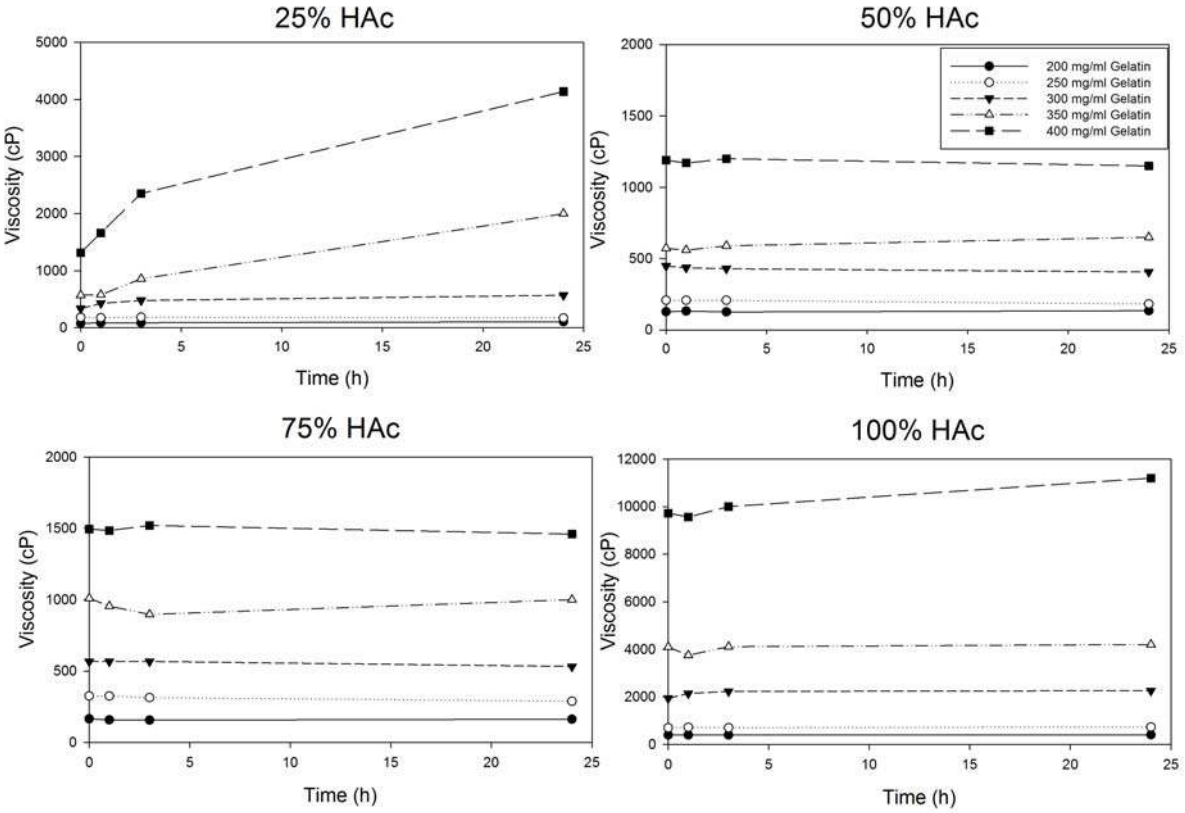

Figure 1. Viscosity changes with storage time for solutions of different gelatin and acetic concentration. All viscosity values have a standard deviation below 2%.

From a practical point of view, these results suggest that performing the electrospinning immediately after dissolving gelatin would be preferable in order to avoid the gelation process. Even so, it is important noting that sometimes a slight gelling process is unavoidable even at this moment, probably because gelation already starts during the long dissolution process due to the combination of high water content and high gelatin concentration. This is the case for those samples made 25% of HAc, which sometimes showed slightly higher viscosities (for 350 and 400 mg/ml) than the obtained for the solutions at the same gelatin concentration but with a higher concentration of HAc (e.g. 50%). The corresponding viscosities are summarized in **Table 1**. It is noteworthy to mention that the viscosity values obtained for the pure acetic acid solutions were in close agreement to those reported previously by Choktaweesap et al. for gelatin solutions with concentrations in the range of 20 - 30% (200 - 300 mg/ml).[26]

Table 1. Viscosity (cP) of the gelatin solutions at time 0 h as a function of gelatin concentration and acetic acid concentration. All viscosity values have a standard deviation below 2%.

		[Acetic acid] (% v/v)			
		100	**75**	**50**	**25**
[Gelatin] (mg/ml)	**200**	395	166	128	77
	250	704	325	208	183
	300	1940	567	448	338
	350	4100	1010	573	574
	400	9725	1495	1190	1317

3.2. Electrospinnability of gelatin solutions

The electrospinnability of gelatin solutions was examined by analyzing the morphology of electrospun fibers by SEM. Obtained results (**Figure 2**) confirmed the feasibility of electrospinning gelatin solutions with high acetic acid concentration combined with low gelatin concentration, previously reported by other authors.[39,42] In this case the high acetic acid content promotes the interaction of gelatin with acetic inducing an increase of the viscosity of the solution, since the viscosity increases as the pH decreases[49], reaching the value of viscosity necessary for electrospinning. What is more relevant, electrospun fibers can also be obtained for low acetic acid concentrations (25% v/v) combined with high gelatin concentrations (>300 mg/ml). In this latter case, the concentration of gelatin is high enough to induce the necessary viscosity and polymer chain entanglement for adequate electrospinning. At the same time, the acetic acid content is high enough to provide electrical conductivity and, most important, to dissolve gelatin avoiding gelation (note that gelation occurs for gelatin in pure water and it absolutely impedes electrospinning).

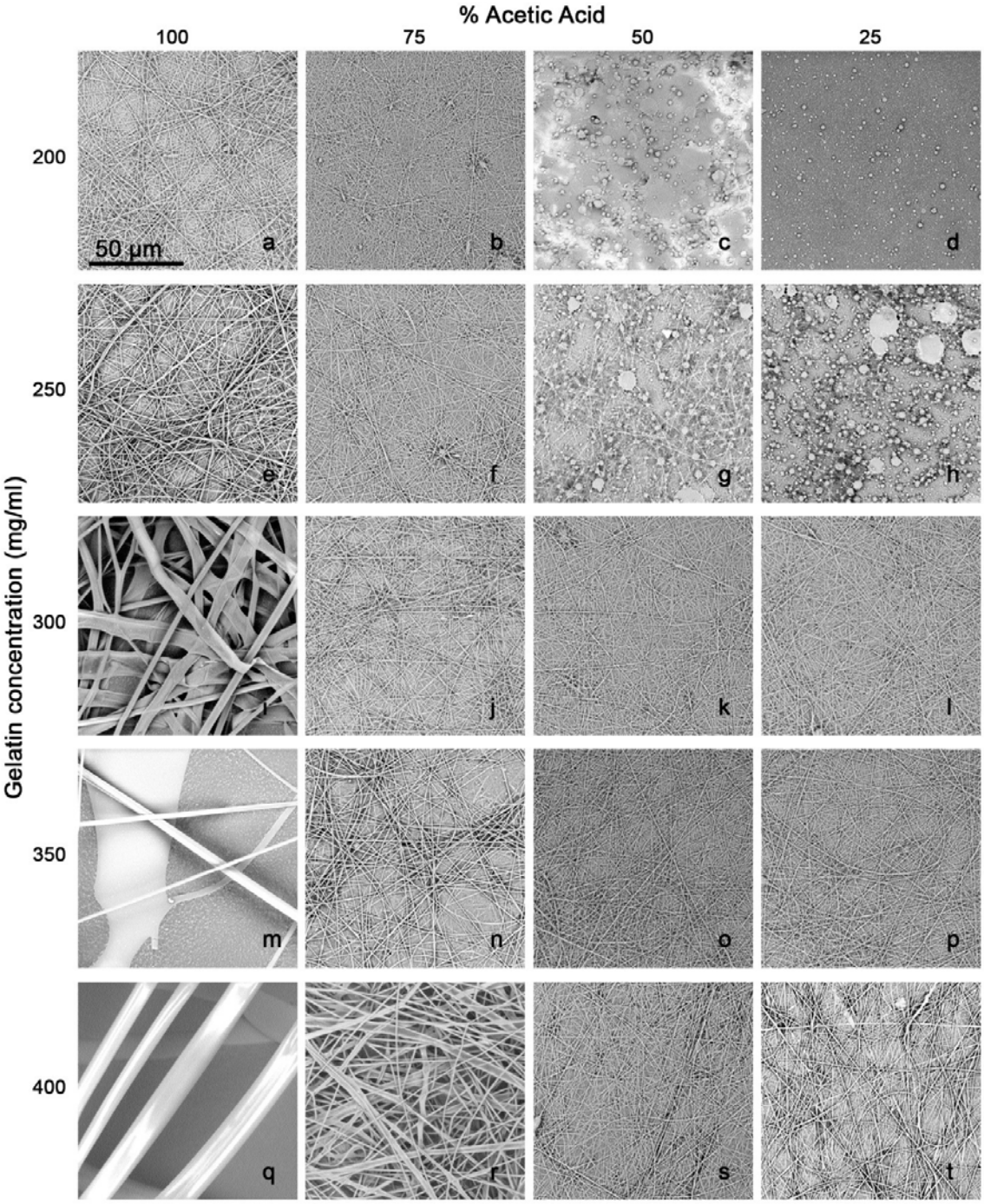

Figure 2. SEM images of electrospun gelatin fibers. The scale bar shown in microphotography a) is valid for all the images.

On the contrary, other combinations were not suitable for electrospinning since they produced either very thick fibers (high acetic acid concentration and high gelatin concentration: Figures 2i, 2m, 2q and 2r) or did not produce fibers at all (low acetic acid concentration and low gelatin concentration: Figures 2c, 2d and 2h). In the first case the viscosity of the solution is very high due to the high amount of gelatin and the acetic acid is only able to partially solvate it, just allowing the electrospinning of very thick fibers or microfibers[43]. In the second case the solutions did not reach the necessary viscosity and polymer chain entanglement to be electrospun. The characterization of some solutions (Figures 2b and 2g), which were partially able to produce nanofibers, also revealed the existence of beads, either as discrete beads or as beaded fibers due to fibers fusion at touching points. Similar behavior has been previously reported for gelatin solutions with a concentration between 200 mg/ml and 300 mg/ml using pure acetic acid as a solvent.[26]

3.2.1. Effect of gelatin concentration

It is obvious from the results that the concentration of gelatin directly affects the viscosity of the mixture (**Table 1**), what is in agreement with the literature.[22,26,50] The viscosity increased along with the gelatin content due to the high chain entanglement between the polymer chains induced by the increase of the polymer concentration. For homogeneous solutions of a linear polymer, the well-known Huggins equation describes this dependence of the solution viscosity with the concentration.[51]

$$\frac{\eta_{sp}}{c} = [\eta] + K_H [\eta]^2 c \quad \text{(Eq 1)}$$

where η_{sp} is the specific viscosity of the polymer solution, $[\eta]$ is the intrinsic viscosity and K_H is the Huggins constant.

For practical purposes, it is important to obtain an optimum viscosity, neither too low so that the fibers cannot be formed (avoiding electrospray and beads-on-string structures) nor so high as to avoid stretching of the solution due to its high molecular weight (solution gelation hinders electrospinning). After having seen how the viscosity influence the electrospinnability of the gelatin fibers, it appears that the fabrication of

gelatin electrospun fibers with a reproducible pattern was constrained to an operational window of viscosity in the range 200-1500 cP (mPa·s) which should coincide with suitable surface tension values as suggested by Geng et al.[46]

In fact, most studies[22,40] recommend to work with low gelatin concentration (lower than 120 mg/ml) at room temperature to avoid the gelation process and to facilitate the electrospinning. Our results showed that the viscosity of gelatin solution strongly depends on the percentage of acetic acid and it is possible and sometimes necessary, to work with high gelatin concentration at which gelation virtually does not occur.

The effect of gelatin concentration on the diameter of the electrospun fibers was further studied (**Figure 3**). The tendency to increase the viscosity of the solution and consequently the diameter of the obtained fibers was maintained, disregarding the exact content of acetic acid in solution. It is therefore concluded that the diameter of fibers is directly related to the viscosity of the spinning solution used, as it has been previously observed for different polymer/solvent systems.[40,52,53]

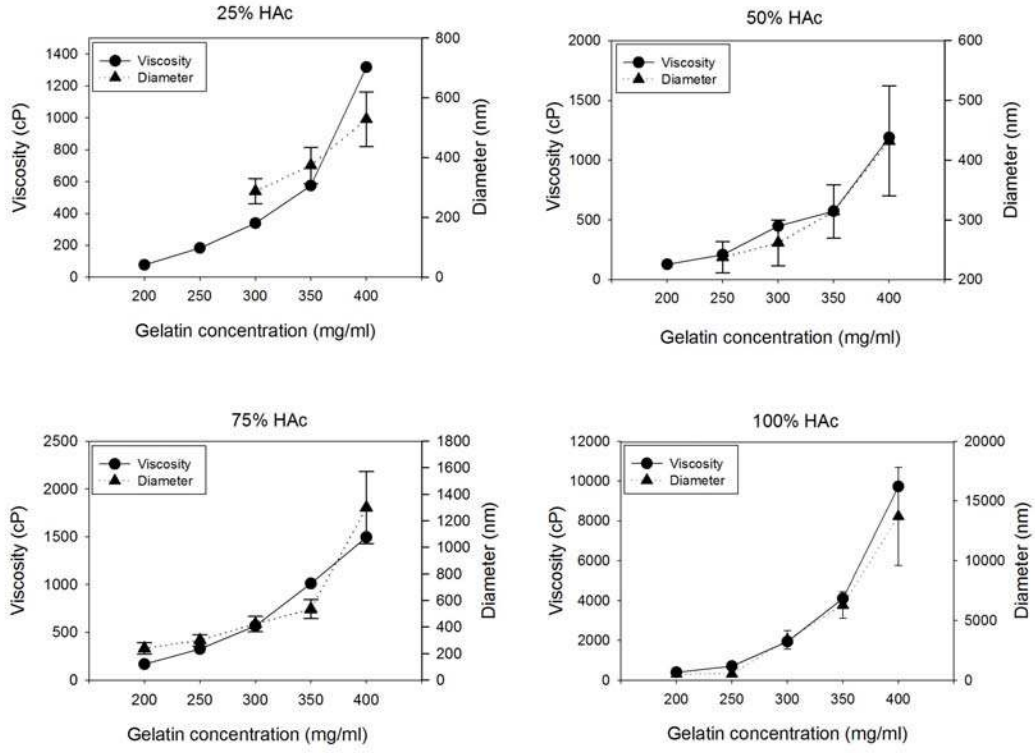

Figure 3. Evolution of the viscosity of solutions and the average diameter of electrospun fibers (with its standard deviation) obtained for each gelatin concentration. All viscosity values have a standard deviation below 2%. Electrospray was obtained at 200 and 250 mg/ml for 25% acetic acid solution and for this reason the diameter were not measured.

3.2.2. Effect of acetic acid concentration

It is well known that the presence of acetic acid influences the surface tension of solutions in a way that the surface tension could be reduced by increasing acetic acid concentration.[22] In this sense, one may expect that viscosity is also affected in a similar way but it is necessary to establish the exact relationship between both parameters, which concern electrospinnability.

Taking into account the experimental findings, at low concentrations of gelatin the acetic acid content did not affect significantly the viscosity. Accordingly, in these cases the electrospinning process is dominated by the surface tension. High surface tension (low acetic concentration) caused the formation of beads, in agreeement with the behavior reported in literature[46,54,55] (see **Figure 2** for solutions containing 25 and 50% v/v acetic acid combined with low concentration of gelatin, i.e. 200 or 250 mg/ml.)

Conversely, increasing the gelatin concentration the effect of acetic acid concentration on viscosity is more noticeable and viscosity is the key parameter that controls electrospinnability, allowing solutions with low acetic acid concentration to spin. Yet, when viscosity was 1500 cP or higher, large diameter fibers with flawed distribution were obtained (see **Figure 2** for solutions with 350 and 400 mg/ml gelatin and high concentration of acetic acid).

It is also important to note that the acetic acid content influences, in turn, the water content and thus, the gelling process. Performing the electrospinning immediately after dissolving the gelatin would avoid the mostly of the spontaneous increase of viscosity, especially for solutions with high content of water (25 % acetic acid).

Similarly to the gelatin concentration, the effect of the acetic concentration was correlated to the fiber diameter (**Figure 4**), corroborating the same behavior which has been observed for gelatin in other solvents.[39,40] In similar way to what happened

with the viscosity trend, the diameter of the obtained electrospun fibers also follows a clear trend and this effect was observed independently of the gelatin concentration, thereby confirming the direct correlation between diameter of the fibers and the viscosity of the spinning solution.

Moreover, the influence of both working parameters (gelatin and acetic acid concentrations) on the viscosity of the spinning solution was statistically analyzed (software package Statgraphics Centurion XV, StatPoint, Inc., USA). The results from an ANOVA test with 95% of uncertainty (**Table 2**) determined that not only both parameters rule the solution viscosity (and, consequently, the diameter of electrospun fibers) but their interaction is also significant ($p = 0.0000 < 0.05$).

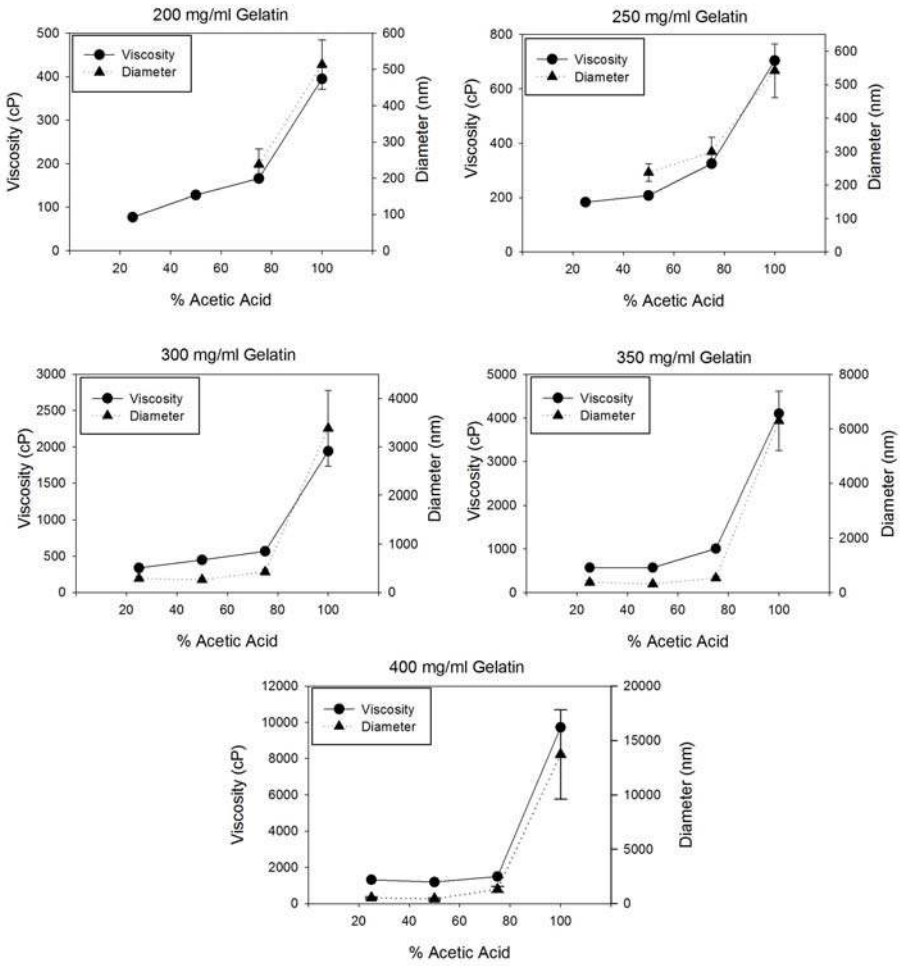

Figure 4. Viscosity of solutions and average diameter of electrospun fibers (with its standard deviation) as a function of the acetic acid concentration. All viscosity values have a standard deviation below 2%.

Table 2. Analysis of gelatin concentration and acid acetic concentration effect on the viscosity of the spinning solution by multifactorial ANOVA.

Source of variance	Sum of Squares	Degrees of freedom	Mean square	F	Critical value of F (p=0.05)
[Acetic acid] (% v/v) (A)	$8.27 \cdot 10^7$	3	$2.91 \cdot 10^7$	1356.36	0.0000
[Gelatin] (mg/ml) (B)	$8.94 \cdot 10^7$	4	$2.23 \cdot 10^7$	1042.27	0.0000
A-B Interaction	$9.81 \cdot 10^7$	12	$8.18 \cdot 10^6$	381.35	0.0000
Error	857502	40	21437.5		
Total	$2.76 \cdot 10^8$	59			

3.3. Electrospun fibers characterization

3.3.1. FTIR

Samples prepared with a fixed gelatin concentration were analyzed by FTIR to determine the effect of acetic acid concentration on the chemical structure of the dissolved gelatin and of the electrospun fibers. Taking into account that a higher concentration of gelatin results in a high effect of the acetic acid on the solution properties[43] it was decided to use the highest gelatin concentration (400 mg/ml) to maximize and improve the characterization of the structural changes in solution state. However, for the characterization of electrospun gelatin mats, a concentration of 300 mg/ml was chosen due to the better electrospinnability of the dope solutions.

The characteristic gelatin IR bands are: amide I, II and III. Amide I (1650 cm^{-1}) is related to C=O stretching vibration coupled with the C-N stretch, amide II (1540 cm^{-1}) arises from out-of-phase combination of C-N stretch and in-plane N-H deformation modes, and amide III (1234 cm^{-1}) reveals the combination peaks between C-N stretching vibrations and N-H deformation from amide linkages as well as absorptions arising from wagging vibrations from CH_2 groups from close amino acid residues.[56] On the other hand, the typical bands for glacial acetic acid are 1706 cm^{-1} (C=O stretching), 1271 cm^{-1} (C-O stretching), 1388 cm^{-1} (CH_2 scissors deformation) and, finally, 3020 cm^{-1} and 2645 cm^{-1} (C-H asymmetric stretching).[57]

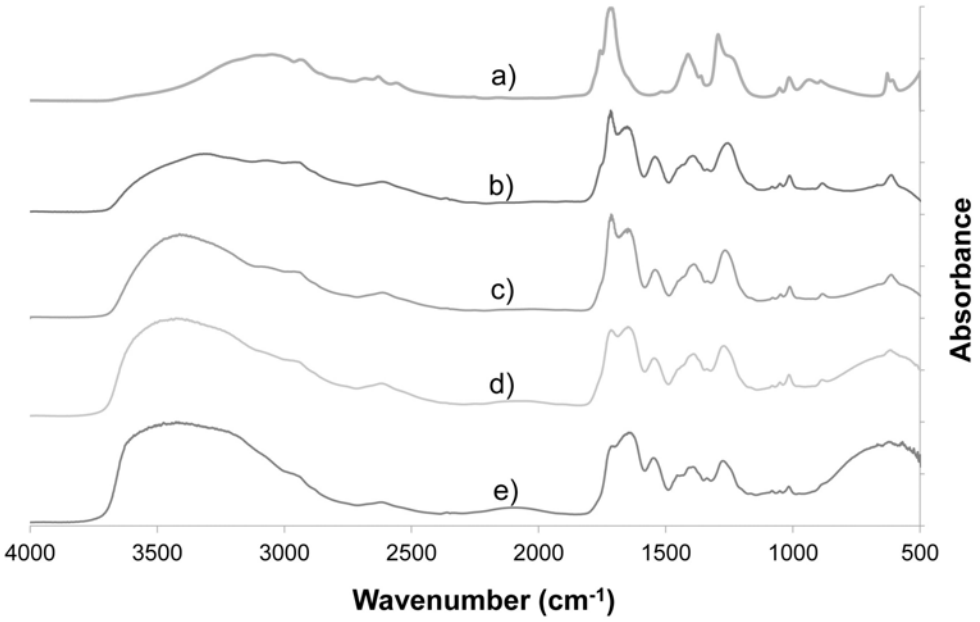

Figure 5. FTIR spectra of gelatin solutions (400 mg/ml) at different acetic acid concentration (% v/v); a) glacial acetic acid b) 100% c) 75% d) 50% e) 25%.

Figure 5 shows the FTIR spectra of gelatin solutions prepared using solvents with different acetic acid concentration and pure acetic acid. It was observed that the IR spectra of the four samples prepared with solutions of increasing concentrations of acetic acid (25% (Figure 5e), 50% (Figure 5d), 75% (Figure 5c) and 100% (Figure 5b)) are an overlapping of the characteristic spectral bands of both gelatin and acetic acid. Additionally, the relative intensity of the characteristic peaks of acetic acid (1706 cm^{-1} and 1271 cm^{-1}, Figure 5a) increased proportionally when increasing the acetic acid content of the electrospinning solutions. Accordingly, the relative absorption peak was minimum for the solution prepared with 25% of acetic acid as solvent (Figure 5e) while the maximum was observed for those solutions prepared with pure acetic acid (Figure 5b)**.**

On the other hand, none of the characteristic peaks of the acetic acid were detected in the FTIR spectra of the electrospun fibers specimens prepared by electrospinning of the abovementioned solution (**Figure 6**), except in the spectra of gelatin electrospun mats fabricated with 100% v/v acetic acid as solvent, where a slight

change in shape of Amide I, suggesting the appearance of a new peak about 1702 cm^{-1}, related with the presence of residual acid. Despite this, the results corroborate the difficulty to detect the remaining acetic acid in the electrospun fibers by FTIR. Yet, FTIR might not be accurate enough to confirm the latter conclusion, as it was suggested by Chang et al.[22] and, therefore, we carried out a DSC analysis to check whether the chemical structure of gelatin was affected by acetic acid in solution.

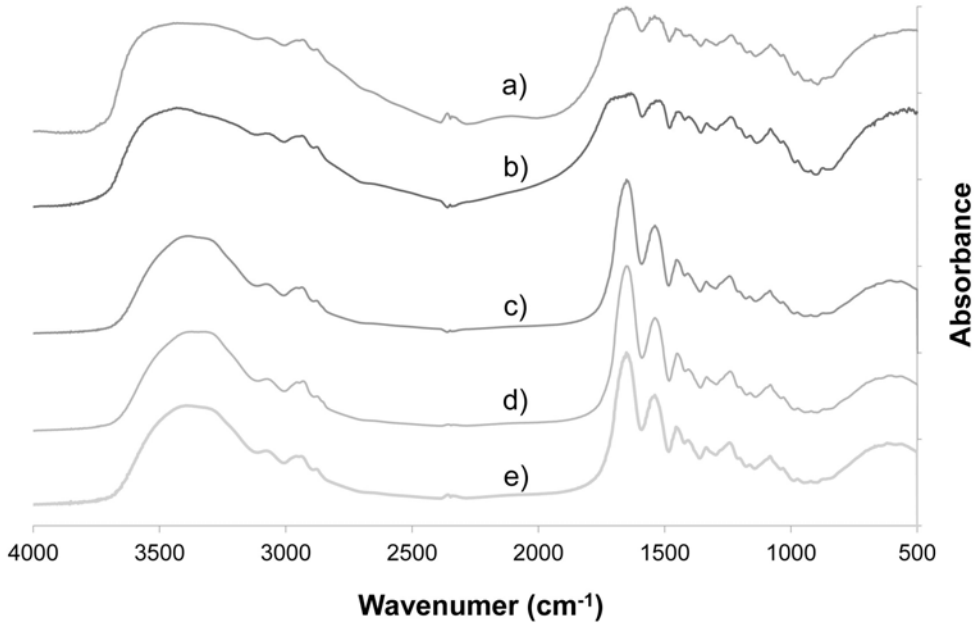

Figure 6. FTIR spectra of electrospun gelatin fibers obtained from solutions containing 300 mg/ml of gelatin and different acid acetic concentration (% v/v); a) powder gelatin b) 100% c) 75% d) 50% e) 25%.

3.3.2. DSC

DSC thermograms of electrospun fibers prepared with solutions containing 25, 50, 75 and 100 % v/v acetic acid and 300 mg/ml gelatin are plotted in **Figure 7**, together with the data corresponding to pure powder gelatin. The peak found about 230 ºC for pure gelatin (Figure 7a) agree with the reported value for gelatin decomposition.[22,40,41] This peak was also found (although slightly shifted) for the nanofiber mats prepared with the lowest acetic acid concentration (25%, Figure 7b)).

Oppositely, in the other solutions (50%, Figure 7c; 75%, Figure 7d; 100% Figure 7e) this peak was not detected, and a wider and shorter peak appeared offset to 200 °C (more deflected at higher acetic acid content). These changes suggest an increase in the amorphous part of the gelatin structure, i.e a decrease in its crystallinity. On the one hand it could be simply explained by the nanoscopic size of fibers, but according with the literature[22], the changes are attributed to alterations of the random coil conformations of the protein.

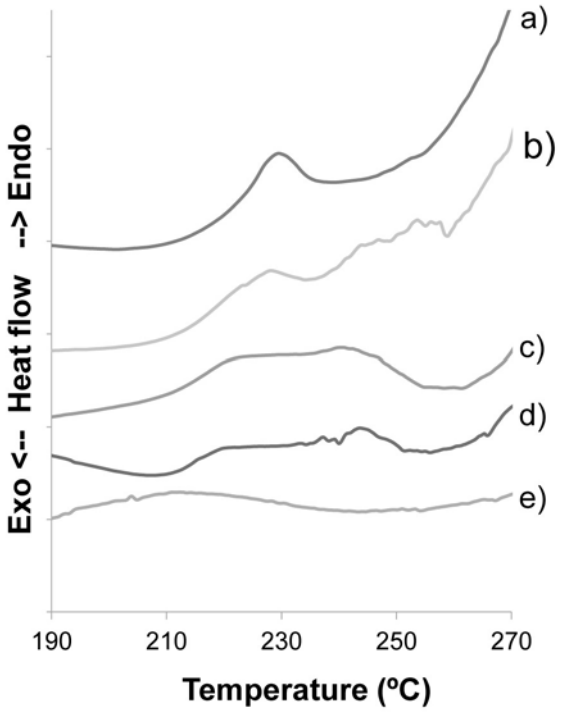

Figure 7. DSC of electrospun fibers obtained for solutions containing 300 mg/ml gelatin and different acetic acid concentrations (% v/v): b) 25 % c) 50 % d) 75 % e) 100 %. Data in a) correspond to powder gelatin.

Thus, despite FTIR spectra did not show many differences for the electrospun fiber mats prepared with different acetic acid concentrations, indicating that the chemical structure of gelatin is not affected, the tertiary structure of the protein is certainly altered causing significant differences in the DSC thermograms. Accordingly, it seems necessary to reduce the acid concentration as much as possible in order to produce nanofibers more analogous to the pristine gelatin.

3.4. Cytotoxicity evaluation

The culture medium used to dissolve the mats of electrospun gelatin fibers contains Phenol Red, a pH indicator frequently used in cell biology that allows for detecting any chemical or microbiological contamination in the medium, which could affect the cells, basing on the color changes. This indicator spans the pH range from 6.8 (yellow) to 8.4 (purple)[58] and is useful to detect any possible trace amounts of acetic acid in fiber mats (**Figure 8**).

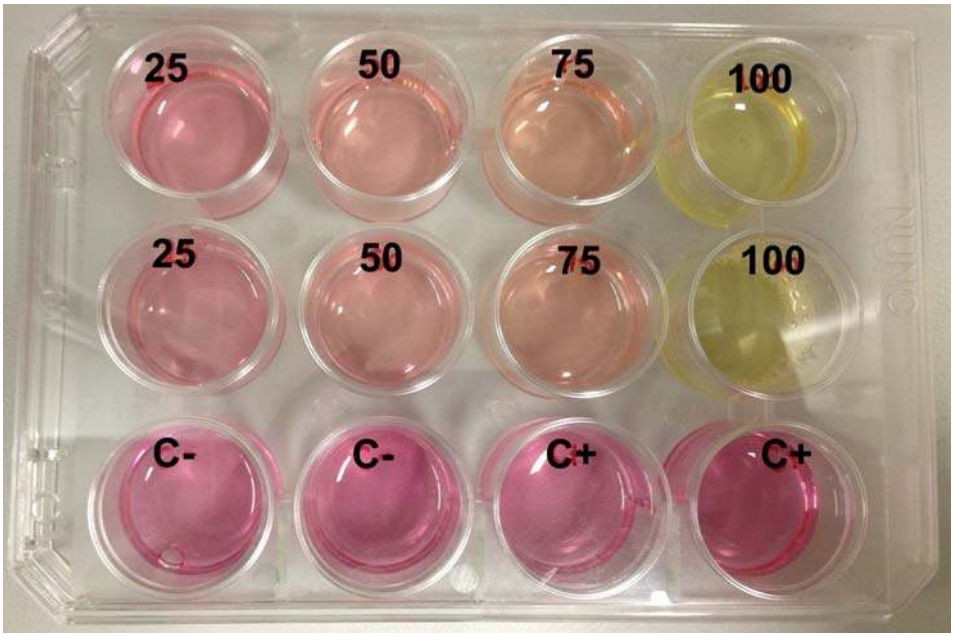

Figure 8. Color changes in culture medium after dissolving the mats of electrospun fibers produced by different acetic acid content solution (25, 50, 75 and 100%). C+ and C- are samples without any electrospun mats dissolved, showing the original color of culture medium, which will used to evaluate the positive and negative control, respectively.

The pH of the cell medium affects the proliferation of skin cells, being the optimal range of pH between 7.2 and 8.3.[59] In this case, the color changes in the medium after exposure to electrospun gelatin fibers fabricated from different acid content solutions were obvious. For the control samples (without gelatin fibers) the color of the medium was pink/purple (4 well below in **Figure 8**), indicating a pH around 7.8 based on the Phenol Red scale, where the proliferation is optimal. Increasing the acetic acid content, the color of medium turned gradually from red (for 25% sample

with pH 7.6) to yellow for 100% acetic acid sample (pH 6), passing through orange for intermediate acid content (50 and 75%) (pH between 7.2 and 7.6). These results confirmed the presence of residual acetic acid in the electrospun gelatin fibers in direct proportion to the acid content in the spinning solution. These observations constituted a preliminary assessment of the toxicity of the developed materials towards the cells.

3.4.1. Alamar Blue assay

To assess the influence of residual acetic acid content in the fibers on the cell viability the Alamar Blue assay was performed on two different types of cells - fibroblasts and HEK (**Figure 9**). The found trend was similar for both cell types: a high cell viability for mats obtained from gelatin solutions with 25% acetic acid content, a slight decrease of cell viability for samples made of 50 and 75% of acetic acid, and a dramatically decrease for a those mats electrospun from solutions of 100 % acetic acid. The confirmed cytotoxicity of the traces of acetic acid contained in gelatin electrospun fibers reinforces the importance of using minimum acetic acid concentration in the future for the electrospinning of gelatin solutions in order to obtain gelatin nanofibers suitable as scaffolds for tissue engineering (i.e. cell viability higher than 90%).

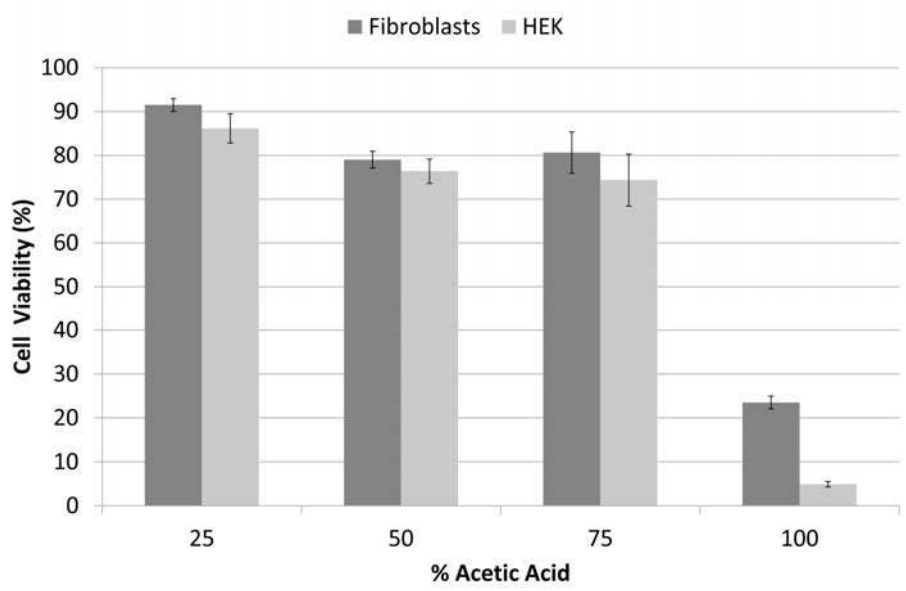

Figure 9. Evaluation of the average cell viability (with the standard deviation) of BJ-5ta fibroblasts and HEK293T cells as a function of acetic acid contained in the electrospun solution.

3.4.2. Cell morphology

The morphology of BJ-5ta fibroblast cells after indirect contact with the different electrospun gelatin fibers was examined after 24 h by phase contrast microscopy (**Figure 10**). The images corroborated the quantitative Alamar Blue assay values, showing low density of cells for the samples that were electrospun with high acetic acid concentration solutions. Moreover, the acid content of electrospun gelatin solutions affected the cell morphology[60] as follows: cells in contact with nanofiber mats formed from solutions with a low concentration of acetic acid had comparable morphology to those displayed in the negative control (elongated, spindle-shape and good attachment), indicating high cell biocompatibility. In contrast, those cells that were in contact with the electrospun fibers obtained from solutions with a high concentration of acetic acid shown a clearly disturbed morphology which was more similar to those cells distributed in positive control (rounded-shape and evidence of cell detachment).

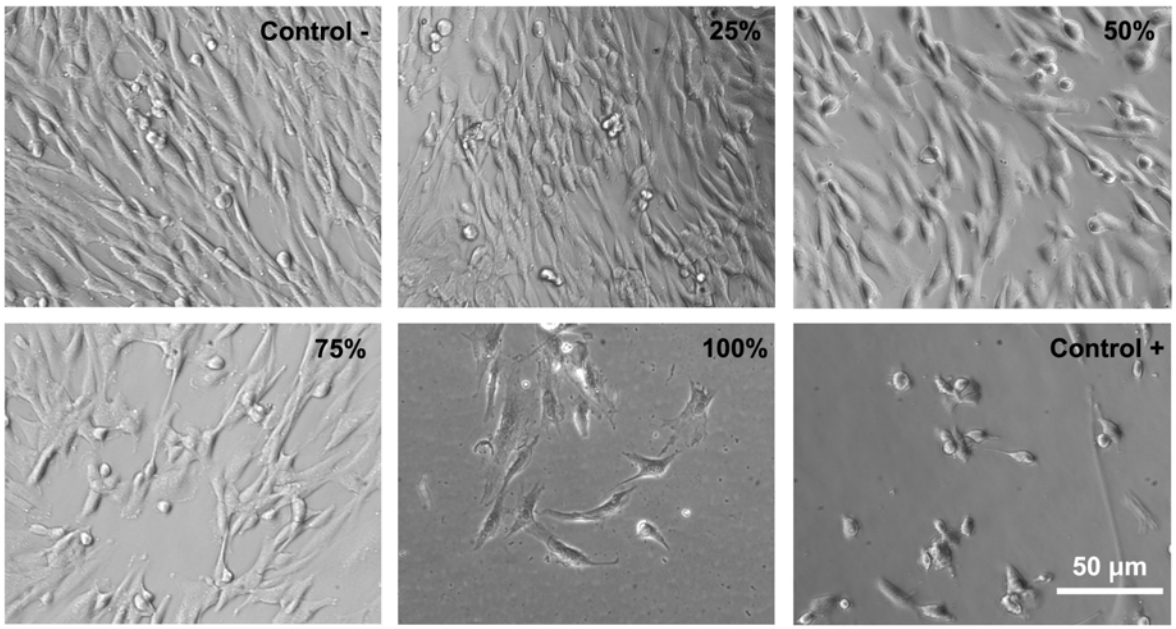

Figure 10. Cell morphology for BJ-5ta fibroblast cells after 24 h in contact with different solutions of mats of electrospun fibers. The scale bar shown in Control + is valid for all the images.

4. CONCLUSIONS

The feasibility of electrospinning gelatin nanofibers from solutions with different concentrations of acetic acid and gelatin at room temperature was tested.

The results showed the viability to obtain electrospun gelatin nanofibers at low acetic acid concentration (25%) combined with gelatin concentration of 300 mg/ml or higher. Both acetic acid content and gelatin concentration exhibited a clear influence on the viscosity solution, which trend was directly correlated with electrospun fiber diameter. Moreover, the study of viscosity solution in front of time determined that the solutions with low acetic acid and high gelatin concentration were those showed the higher rheology instability, due to the gelation process, suggesting the importance to develop the electrospinning just after 1h of stirring the solution.

Although the FTIR spectra did not show many differences on the electrospun gelatin mats in function of acetic content, the DSC analysis allows to determine the benefit to work at low acetic acid concentration, being the electrospun mat from 25% of acetic acid the only sample that keeps showing the characteristic degradation peak of pure gelatin at 230ºC, related with the crystallinity conformation of polymer.

Finally, the indirect cytotoxicity assay demonstrated the direct relationship between the acetic concentration of the solution and the acid traces found in the final mats revealed by the pH indicator changes. Also, the greatest cell viability (upper than 90%) was achieved for mats from solutions at 25% acetic acid concentration.

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