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Development of PVA/gelatin nanofibrous scaffolds for Tissue Engineering via electrospinning

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Abstract

The electrospinning process is an emerging and relatively easy technique to prepare three-dimensional matrices with micro- and nanofibers. To achieve it, aqueous polymer solutions from synthetic or natural polymers are used. PVA was selected as polymer and gelatin because of its biocompatibility and biodegradability. A complete characterization of the polymeric solutions (density, surface tension, etc) was previously performed. Subsequently, a standard electrospinning process (15 kV, 0.4 ml h^{-1} and 10 cm) was carried out to obtain scaffolds. The influence of the polymer concentration and the protein addition was observed by performing FTIR analyses and studied by analyzing the water contact angle and SEM images.

1. Introduction

Tissue Engineering is an interdisciplinary field whose main objective is the functional recovery of tissues. It is based on three main elements: cells, growth factors and scaffolds. The scaffold is one of the most important elements to take into account in the development of an efficient biomaterial for Tissue Engineering. Scaffolds need certain properties to make them suitable, such as pore size and distribution, surface adhesion, biocompatibility and mechanical resistance that allow their mechanical integrity during the sterilization and storage, as well as their degradation and subsequent substitution for the tissue [1].

One of the most emerging techniques for the formation of scaffolds with application in regenerative medicine consists of the fabrication of membranes, formed by nanometric fibers, through electrospinning. In the last few years, an intensive research effort has been developed in the field of processing of polymers through electrospinning [2–5], which reveals the huge potential of this technique for several applications, like modulable hidrophobicity and water adhesion materials [6], air filtration [7], controlled release of drugs [8], biosensors [9], encapsulation of functional food [10], antimicrobial nanofibers development [11] or Tissue Engineering [12]. Thus, nanofibres are appropriate for their use in biomedical applications like wound dressing or tooth materials and, specially, in tissue engineering as scaffolds for tissues regeneration or organs implant [13].

This electrospinning process to obtain fibers presents several advantages to other traditional methods such as the high specific surface and porosity of these fibers. It can be also highlighted the possibility of producing fibers with the desired properties by changing their chemical or structural composition. Furthermore, some characteristics like the fiber diameter, the three-dimensional structure or even the matrix composition can be adjusted by modifying the process parameters: the relation between polymer/copolymer used, the voltage, the flow rate or the distance between the needle and the collector [14]. The use of biopolymers, like proteins, presents the advantages of biodegradability and the ease for crosslinking (giving a suitable mechanical resistance to membranes). All of them are essential for the use of these fibers in Tissue Engineering and, for that matter, in regenerative medicine. The most used proteins are collagen, gelatin, elastin and tropoelastin [15], but gelatin (GE) is selected because of its biocompatibility, which is essential for Tissue Engineering.

However, there is sometimes a drawback related to the relatively low capacity to form fibers of proteins. For that reason, it is necessary the incorporation of another polymer (synthetic polymer) that enables the

electrospinning of polymer/protein solutions. Between the most used synthetic polymers are found polystyrene (PS), Poly(vinyl alcohol) (PVA) and Polycaprolactone (PCL) [16–18]. PVA is a water-soluble polymer which highlights among the others for being non toxic, highly flexible and, which is more important, biodegradable [19].

Most of the studies carried out in this field are focused on the properties of the fibers obtained [20, 21]. Nevertheless, only a few are also focused on the properties of the solutions previously prepared, which are a key factor since they have a great impact on the electrospinning process by modifying the final properties of the fibers obtained. Thus, the novelty of this work lies in the combinatorial study of the properties of the electrospinning solutions as well as the properties of the final scaffolds obtained.

For everything explained previously, the aim of this work is the study of both the polymer solutions prepared using PVA and Gelatin and the final membranes of nanofibers processed by electrospinning with their potential application in Tissue Engineering.

2. Material and methods

2.1. Materials

Gelatin protein (GE) is a fish gelatin type B (80–120 g Bloom) and it was supplied by Henan Boom Gelatin Co. Ltd (China). It presented a protein content of ca. 98 wt% and it was also composed of ash, lipids and moisture with a content of less than 1 wt%. On the other hand, Poly(vinyl alcohol) (PVA, $M_w = 130\,000$ g mol^{-1}; hydrolysis 86.7%–88.7%) was purchased from Sigma Aldrich (Germany). The water was deionized before using.

2.2. Preparation of polymer solutions

Both PVA and GE are water-soluble so different aqueous solutions with PVA and GE were prepared increasing the polymer concentration (named as 2.5 wt%, 5.0 wt%, 7.5 wt% and 10 wt%). Moreover, mixed PVA/GE systems were also prepared varying the PVA-GE ratio (10/0 wt%, 7.5/2.5 wt%, 5.0/5.0 wt%, 2.5/7.5 wt% and 0/10 wt%). All the solutions were prepared with magnetic stirrer during 4 h at 40 °C maintaining constant the amount of solvent present (H_2O) from the beginning of the preparation.

2.3. Physical characterization of solutions

The properties of the solutions affect the electrospinning process. For that reason, a complete study of the previous solutions is performed: electrical conductivity, surface tension, density and viscosity measurements.

2.3.1. Electrical conductivity

The electrical conductivity was measured with an EC-Meter Basic 30 + (Crison Instruments) equipment. All the measurements were determined at 25 °C.

2.3.2. Surface tension

Surface tension of the different solutions was measured using a Sigma 701 tensiometer based on the Wilhelmy method. The temperature was maintained at 25 °C with a thermostat.

2.3.3. Density

The density was obtained at 25 °C by means of a Densito 30PX Portable Density Meter (Mettler Toledo).

2.3.4. Viscosity

Viscosity of gelatin solutions was measured with an Ubbelohde glass capillary viscometer (Proton; size Inc., Barcelona, Spain). Viscosity measurements of PVA and PVA/GE solutions were carried out by means of an AR2000 (TA Instruments, New Castle, DE, USA) rheometer. All the flow curves from 1 to 200 Pa were carried out at 25 °C (controlled by a Peltier connected to a thermostatic bath) using a 40 mm plate-plate geometry. In order to decrease the possible inertia and to avoid slides, aluminum serrated plates were used.

Besides, according to the needle (gauge 22) and the flow rate (0.4 ml h^{-1}) used for electrospinning, the theoretical shear rate inside the capillary where the solution is projected is 9.1 s^{-1} (calculation below):

For a Newtonian fluid, the shear rate is calculated by the following relation:

$$\dot{\gamma} = 8D/u$$

Where D is the diameter of the needle (gauge 22, diameter $5{\cdot}10^{-4}$ m) and u is obtained as follows:

$$u = Q/A$$

Where Q is the flow rate (0.4 ml h^{-1}) and A is the circular area ($\pi \cdot r^2$).

Table 1. Viscosity values of the different systems studied (PVA, GE and PVA/GE) at 25 °C. Values with different letters are significantly different ($p \leqslant 0.05$).

POLYMER SOLUTIONS		
Systems		Viscosity at 10 s^{-1}(Pa · s)
PVA	10 wt%	1.49 ± 0.18^{a}
	7.5 wt%	$4.05{\cdot}10^{-1} \pm 1.50{\cdot}10^{-1\,b}$
	5.0 wt%	$1.26{\cdot}10^{-1} \pm 3.72{\cdot}10^{-2\,c}$
	2.5 wt%	$1.03{\cdot}10^{-2} \pm 2.55{\cdot}10^{-3\,d}$
GE	10 wt%	$2.29{\cdot}10^{-3} \pm 7.20{\cdot}10^{-5\,A}$
	7.5 wt%	$1.54{\cdot}10^{-3} \pm 4.69{\cdot}10^{-4\,B}$
	5.0 wt%	$1.38{\cdot}10^{-3} \pm 5.60{\cdot}10^{-5\,B}$
	2.5 wt%	$6.61{\cdot}10^{-4} \pm 2.77{\cdot}10^{-4\,C}$
PVA/GE	10/0 wt%	$1.49 \pm 0.18^{\alpha}$
	7.5/2.5 wt%	$5.66{\cdot}10^{-2} \pm 1.52{\cdot}10^{-2\,\beta}$
	5.0/5.0 wt%	$2.55{\cdot}10^{-2} \pm 9.60{\cdot}10^{-3\,\gamma}$
	2.5/7.5 wt%	$3.51{\cdot}10^{-3} \pm 7.00{\cdot}10^{-4\,\delta}$
	0/10 wt%	$2.29{\cdot}10^{-3} \pm 7.20{\cdot}10^{-5\,\varepsilon}$

For that reason, the viscosity results shown in table 1 (obtained from the flow curves for PVA and PVA/GE solutions) correspond to 10 s^{-1}.

2.4. Electrospinning process

The electrospinning process is relatively innovative and allows the formation of nanometric and micrometric fibers. It is based on the deformation of a drop from a polymeric solution to form a mat. The mat is formed due to an electric voltage field, which produces electrostatic repulsions between charged surfaces. The drop produced has a conical shape, obtaining the named *Taylor's cone* and it is projected from a syringe (connected to a needle where the polymeric solution is) to a collector, where the nanofibrous mat is formed. To produce the nanofibrous scaffolds, some processing parameters should be optimised, thus, the conditions selected as reference for the processing of the different electrospun nanofiber systems should be: an intermediate voltage in order to form the Taylor's cone [22], and a flow rate and a needle-collector distance enough to produce suitable fibers but not so high because it would produce flaws in the fibers [16].

Firstly, the solutions were stirred at room temperature for 4 h, being stable during the mixing and the electrospinning process at the working temperature. Following to the stirring, the electrospinning process was performed at 15 kV using a 10 ml syringe with 22 G stainless steel needle (inner diameter 0.5 mm), with a flow rate of 0.4 ml h^{-1} and a needle-collector distance of 10 cm.

2.5. Characterization of nanofibrous scaffolds

2.5.1. Fourier transform infrared spectroscopy (FTIR)

The chemical bonds were analysed by ATR-FTIR method using an iS50 ATR-FTIR spectrophotometer (Nicolet). The different spectra were collected in the range of 4000–500 cm^{-1}.

2.5.2. Water contact angle (WCA)

Scaffolds wettability and hydrophobicity were assessed by water contact angle (WCA) measurements using the sessile drop method (droplets with an approximate volume of 5 μl). Both WCA values of the right and left sides of the deionized water droplets were measured and the average value was calculated. The equipment used was a Drop Shape Analyzer (Krüss).

2.5.3. Scanning electron microscopy (SEM)

Microscopy examination of scaffolds has been assessed with a JEOL JSM 6460 LV (Tokyo, Japan) at an acceleration voltage of 25 kV and a magnification of 2000x. SEM images were obtained using the own software of the equipment. In addition, the morphology of the fibers was studied using a digital processing software (ImageJ). The mean diameter has been obtained by measuring several fibers of three different images of each systems.

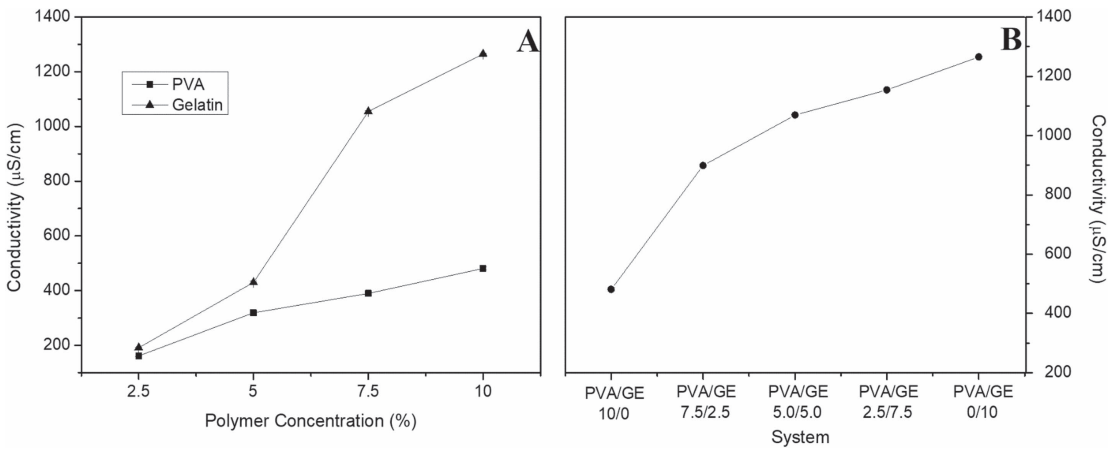

Figure 1. (A) Conductivity values of the PVA and Gelatin (GE) aqueous solutions as a function of the polymer concentration (0, 2.5, 5, 7.5 and 10 wt%) and (B) Conductivity values of the mixed systems PVA/GE studied (10/0, 7.5/2.5, 5.0/5.0, 2.5/7.5, 0/10).

2.6. Statistical analysis

At least three replicates were carried out for each measurement. Statistical analyses were performed with t tests and one-way analysis of variance ($p < 0.05$) using PASW Statistics for Windows (Version 18: SPSS, Chicago, IL). Standard deviations were calculated for selected parameters.

3. Results and discussion

3.1. Physical characterization of GE, PVA and PVA/GE solutions

A control of the properties of the solutions is important to evaluate the potentiality of electrospinning for obtaining fibers with suitable morphology and size, provided that other parameters are also controlled like voltage, flow rate, temperature or humidity at which the process takes place. Thus, conductivity, surface tension, density and viscosity of the solutions were measured.

Figure 1 shows the evolution of conductivity with the concentration of polymer (figure 1(A)) and the conductivity values of the mixed systems (figure 1(B)). As may be observed, the conductivity of these aqueous solutions increases with the concentration of polymer and biopolymer (protein), being more significant for the gelatin solutions (10 wt% gelatin solution presents a three times higher conductivity than 10 wt% PVA solution). Thus, the electrical conductivity of the mixed systems is higher when the concentration of gelatin is higher, which is positive because it allows a better charge transport and, thereby, a better electrospinning process [16].

Considering surface tension values, PVA and gelatin aqueous solutions (figure 2(A)) present no significant differences and remain relatively constant with concentration (ca. 45–46 and 55 mN m^{-1}, respectively). That means that gelatin, despite being a protein, present a lower surface activity than PVA. Respect the mixed systems, the values shown in figure 2(B) demonstrate that these values are in the range of PVA solutions (between 45 and 47 mN m^{-1}), so PVA control the values for surface tension due to its higher surface activity. A high surface tension would produce defects in the fibers (instability) so low surface tension values are recommended [16].

As may be observed in figure 3(A), the density of both PVA and GE solutions increase with polymer concentration, showing similarities between the values obtained for both polymers. The study of the mixed systems (figure 3(B)) reveals that all the solutions have no significant differences compared to the values obtained for the solutions prepared with a 10 wt% of PVA or GE.

Table 1 shows the values of viscosity for the PVA and GE aqueous solutions, as well as the mixtures of PVA/GE studied. The gelatin solutions exhibit a slight increase when the concentration of gelatin present is higher. Moreover, the PVA solutions present a similar shear-thinning behaviour (data not shown) with a slight decrease in viscosity along the shear rate increases, although a terminal viscosity is achieved (η_∞) from 5 s^{-1}. According to the results shown in table 1, an increase in PVA concentration also produced an exponential increase on the viscosity of these solutions. However, the PVA/GE solutions present intermediate viscosities (lower than PVA solutions but higher than GE ones), which may be suitable to be processed by electrospinning because a high viscosity would affect the fiber size or, even, would obstruct the needle.

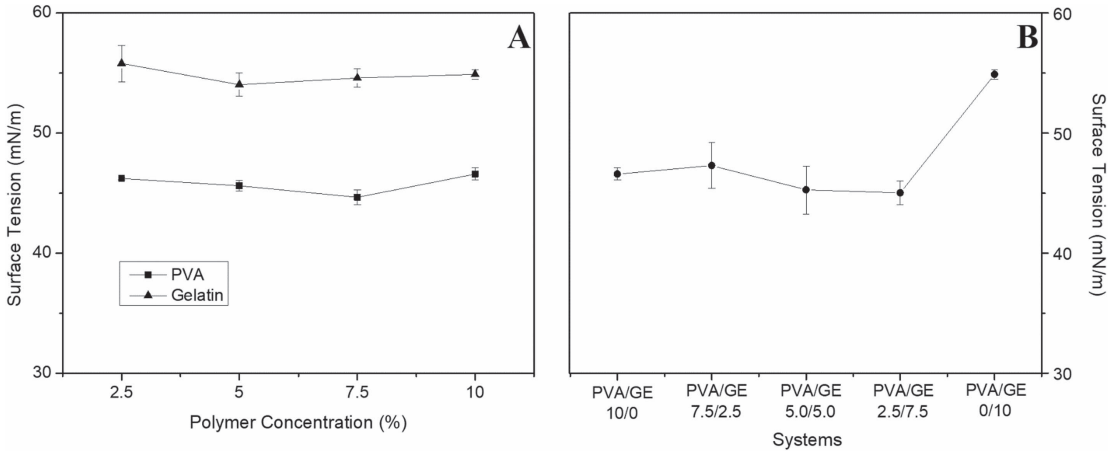

Figure 2. (A) Surface tension of the PVA and Gelatin (GE) aqueous solutions as a function of the polymer concentration (0, 2.5, 5, 7.5 and 10 wt%) and (B) Surface tension of the mixed systems PVA/GE studied (10/0, 7.5/2.5, 5.0/5.0, 2.5/7.5, 0/10).

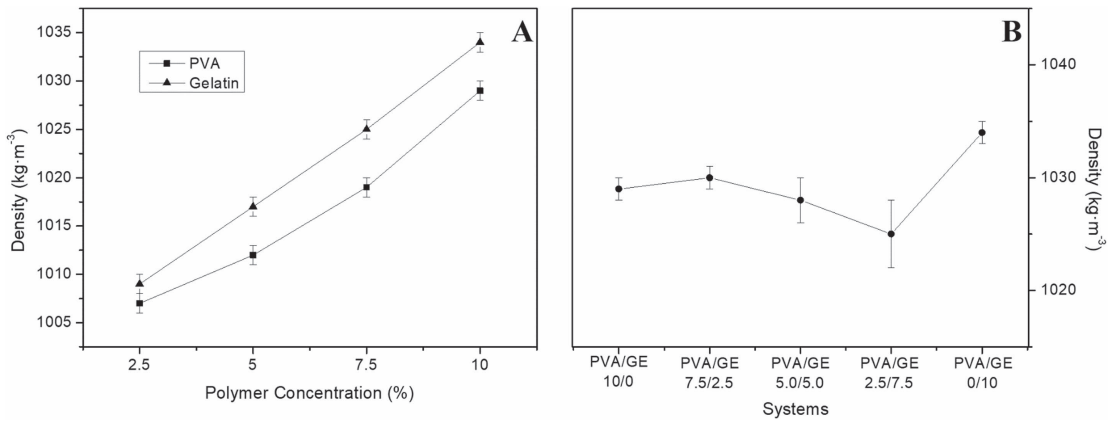

Figure 3. (A) Density of the PVA and Gelatin (GE) aqueous solutions as a function of the polymer concentration (0, 2.5, 5, 7.5 and 10 wt%) and (B) Density of the mixed systems PVA/GE studied (10/0, 7.5/2.5, 5.0/5.0, 2.5/7.5, 0/10).

3.2. Characterization of nanofibrous scaffolds

3.2.1. Fourier transform infrared spectroscopy (FTIR)

To follow the evolution of gelatin in the scaffolds produced, a FTIR analysis of three of the systems studied was performed (PVA/GE 10/0 wt%, PVA/GE 5.0/5.0 wt% and PVA/GE 0/10 wt%). Thus, FTIR spectra for these samples are shown in figure 4. Two different profiles can be seen comparing the spectra obtained for the system with only PVA (straight line) or Gelatin (bold line). The system obtained with PVA (PVA/GE 10/0 wt%) shows a broad peak at ca. 3400 cm^{-1} characteristic for the O-H group and two peaks at 2900 cm^{-1} which are referred to the stretching of C-H. In addition, there are other peaks at 1400–1350, 1096 and 830 cm^{-1} for CH_3 symmetrical deformation, C-O and CH_2, respectively [23]. On the other hand, the spectrum exhibited by the PVA/GE (0/10 wt%) system shows the characteristic peaks for gelatin, highlighting a broad area at ca. 3400 cm^{-1} associated to N-H stretching (Amide A signal), bands at 1635 and 1525 cm^{-1} related to C=O and C-N stretching of amides, and a band at ca. 1240 cm^{-1} for N-H bending. In addition, this spectrum also presents the bands related to the CH_2 symmetrical and asymmetrical stretching and bands in the range 1450–1000 cm^{-1} (C-H bending and wagging) but with a lower intensity [24]. Interestingly, the profile exhibited by the system produced with a mixture of both polymers show a combination of both spectra (dash line), highlighting the characteristic bands of proteins at 1635 and 1524 cm^{-1} and the proper bands of PVA at 1096 and 830 cm^{-1} (all of them with a lower intensity than the unitary systems).

3.2.2. Water contact angle (WCA)

Figure 5 shows the water contact angle of the five systems studied and the images of the droplet cross-section for each system. It can be highlighted how the increase in gelatin content produces scaffolds with a lower WCA, going from 50° (PVA system) to 10° (gelatin system). It is interesting to point out how a small amount of gelatin

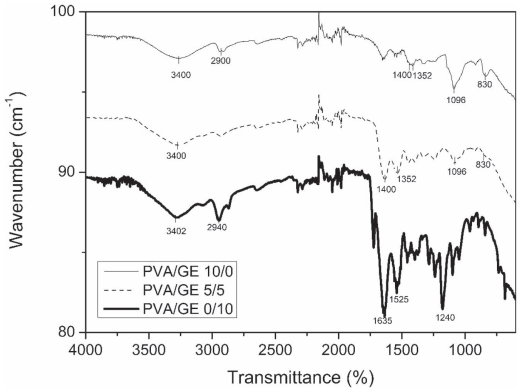

Figure 4. FTIR analysis of PVA/GE (10/0 wt%), PVA/GE (5.0/5.0 wt%) and PVA/GE (0/10 wt%).

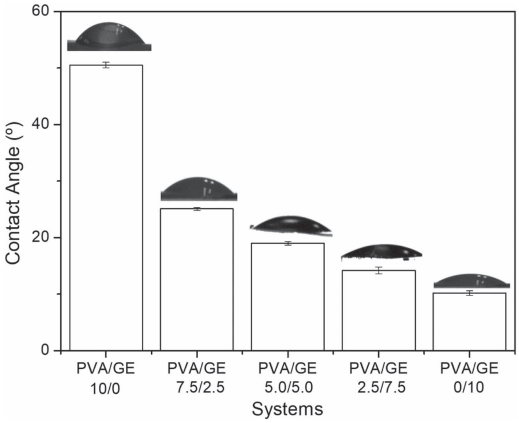

Figure 5. Water contact angle of scaffolds obtained from the mixed systems PVA/GE studied (10/0, 7.5/2.5, 5.0/5.0, 2.5/7.5, 0/10). An image of the droplet cross-section of each system has also been included. Values with different letters are significantly different ($p \leqslant 0.05$).

(2.5%) produces a marked decrease of the WCA of the resulting scaffold (from 50° to 25°), obtaining more hydrophilic scaffolds.

3.2.3. Scanning electron microscopy (SEM)

Figure 6 shows the SEM images of electrospinning mats obtained as a function of PVA content (10, 7.5, 5.0 and 2.5 wt%). As it can be seen in these images, a decrease in PVA concentration leads to an increase in the amount of fibers formed but with a lower diameter, as it can be seen in table 2.

This decrease could be produced to the decrease in the viscosity of the solution used during the process (which varies with the amount of PVA present). However, the uniformity of the nanofibers is more irregular when the concentration of PVA is lower than 5 wt%. This effect is related to the elimination of solvent during fibers processing, because the system used is less and less concentrated. For that reasons, the more suitable morphological characteristics correspond to the systems with an intermediate PVA concentration (5.0 and 7.5 wt%).

On the other hand, figure 6 also shows the evolution of the electrospun fibers with the addition of protein (gelatin protein) in increasing concentrations (0, 2.5, 5.0, 7.5 and 10 wt%) but maintaining constant the total concentration of polymer (10 wt%). It is important to highlight that a decrease in PVA an evolution from a homogeneous matrix towards a matrix formed by spheroidal microparticles called beads, which are connected through nanofibers.

The results suggest that the protein is encapsulated inside these spheroids, with a wrap of PVA that extends forming nanofibers connecting the different spheroids. This effect is related with the increase in electrostatic charges [25]. Besides, the amount of particles produced increase with the replacement of PVA by GE, for that matter decrease the number of nanofibers formed. Interestingly, a similar behaviour it is also observed using a globular protein as whey protein (data not shown). Furthermore, when the concentration of gelatin present is higher than 50%, the electrospinning process is not properly fulfilled because no nanofibers are obtained

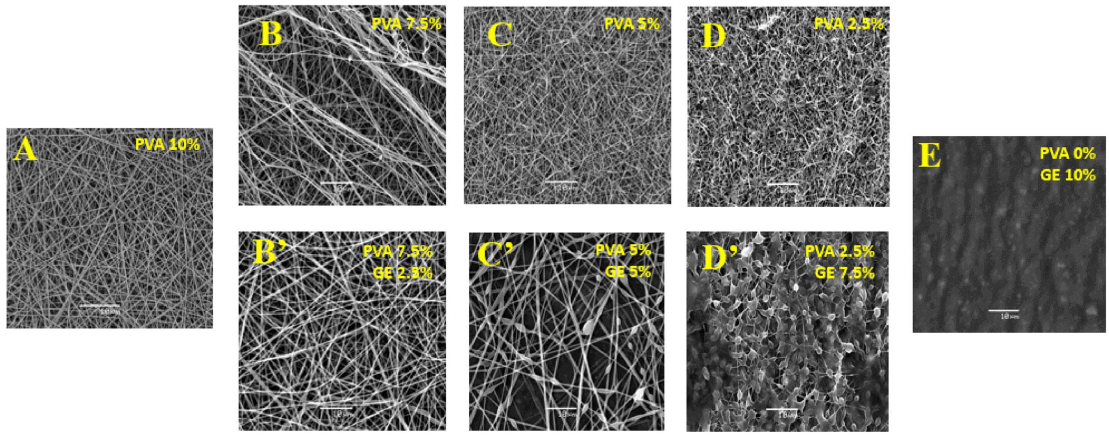

Figure 6. SEM images of scaffolds made from 10 (A), 7.5 (B), 5 (C) and 2.5 (D) wt. % PVA solutions and PVA/Gelatin solutions with different proportions: 7.5/2.5 wt% (B′), 5.0/5.0 wt% (C′), 2.5/7.5 wt% (D′), and 0/10 wt% (E′) at 15 kV, 0.4 ml h^{-1} and 10 cm.

Table 2. Fiber diameter of the nanofibers obtained from the systems processed by electrospinning (PVA and PVA/GE). Values with different letters are significantly different ($p \leqslant 0.05$). * means that nanofibers could not be obtained.

NANOFIBERS		
Systems		Fiber Diameter (μm)
PVA	10 wt%	0.46 ± 0.14^{a}
	7.5 wt%	0.32 ± 0.04^{a}
	5.0 wt%	0.22 ± 0.07^{b}
	2.5 wt%	0.12 ± 0.05^{c}
PVA/GE	10/0 wt%	$0.46 \pm 0.14^{\alpha}$
	7.5/2.5 wt%	$0.30 \pm 0.04^{\beta}$
	5.0/5.0 wt%	$0.41 \pm 0.09^{\alpha\beta}$
	2.5/7.5 wt%	*
	0/10 wt%	*

(electrospraying is achieved instead of electrospinning because it is not processed continuously), in accordance to Sullivan *et al* (2014) [26].

4. Conclusions

As a general conclusion, nanofibrous scaffolds with different PVA/GE ratios have been obtained by electrospinning with a suitable fiber size (so high specific surface) and a microstructure that present a huge potential for their applications in Tissue Engineering.

From the study of the different solution properties (conductivity, surface tension, density and, above all, viscosity) it has been determined that is possible to work with PVA and GE concentrations up to 10 wt% in order to be successful during the electrospinning process.

An increase in the polymer content produces solutions with a higher conductivity, viscosity and density. However, the surface tension is not affected by the concentration of polymer present, remaining constant in all the concentration range studied.

The processing of PVA/Gelatin solutions (with a protein concentration higher than 50%) highlights the impossibility of obtaining nanofibers by electrospinning from these solutions, probably due to the low viscosity shown.

An increase in the concentration of PVA leads to a more regular morphology and fibers with higher sizes. The lack of regularity found with the lowest polymer concentrations is attributed to the greater difficulty in solvent elimination.

PVA/GE matrices in different proportions have been developed to obtain fibers with suitable morphological and structural properties. The replacement of PVA by protein (gelatin, GE) is observed in the FTIR profiles of the

systems, showing the characteristic peaks for one or both polymers. Furthermore, with the aim of increasing the biocompatibility of the systems, the scaffolds produced show a more hydrophilic character and suffer an evolution in the morphology: from a system consisted of cylindrical nanofibers to another consisted of spheroids (beads) interconnected by nanofibers. The frequency of spheroids formation increases with the concentration of protein.

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