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Electrospinning of high concentration gelatin solutions

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Non-woven gelatin nanofiber membranes membranes were prepared by electrospinning of high concentration gelatin solutions. Electrospinnable solutions of 27-30% (w/v) gelatin concentration were prepared using various solvents. The solvent mixture consisting of formic acid, acetic acid and dimethylformamide in 4:0.5:0.5 volume ratio gave the best results, the gelatin solution remaining stable for more than 48 hours, as proved by viscosity measurements. The chemical and physical structure of the gelatin nanofiber membranes were investigated by scanning electron microscopy, infrared spectroscopy, differential scanning calorimetry and X-ray diffraction. The observed chemical and physical properties were compared to those of gelatin films prepared from the same solutions. The films showed structural differences depending on the solvent mixture used, while the characteristics of the electrospun membranes were almost similar.

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1. Introduction

Gelatin is a natural biopolymer with a wide range of applications in medical, pharmaceutical and food industries. In the last years, gelatin based scaffolds prepared by electrospinning were intensely investigated because their three-dimensional (3D) structure that mimics very well the extracellular matrix makes them very attractive for tissue engineering applications [1-5].

The first important step in electrospinning a natural polymer is the preparation of an electrospinnable solution using a proper solvent.

Gelatin is a natural polymer with strong polarity. It has molecular chains connected through strong hydrogen bonds, constituting a 3D macromolecular network (double or triple helix) with reduced mobility [6]. In order to dissolve gelatin, high-polarity solvents are required to break the links between chains and change its structure from helix to random-coil.

Gelatin can be made to undergo reversible helix-coil transformations by choosing appropriate solvent systems. Addition of urea or thiocyanates to aqueous gelatin solutions hinders gelatin renaturation, and treating films of helical gelatin with solutions of urea or thiocyanates results in a transition to the coil structure [7]. Adding crosslinking agents to a gelatin solution may promote or hinder renaturation of gelatin, depending on the relative rates of the helix formation and crosslinking processes [8].

Water is a good solvent of gelatin because it breaks very easy the interchain links and produces stable solutions at 50°C, without gelatin degradation. However, an aqueous gelatin solution changes into a gel in the syringe needle at room temperature and the electrospinning process becomes impossible. Moreover, water has a slow evaporation rate, making impossible the transformation of the solution filament into a dry nanofiber during the travel between needle and collector.

Because of these limitations in using water as a solvent, electrospinning of gelatin requires the use of fast-evaporating organic solvents. Formamide, dimethyl sulphoxide and 2-chloroethanol were used because they prevent helix formation [8]. Fluorinated alcohol solvents such as trifluoroethanol and hexafluoro isopropanol were found also to be good solvents for polypeptides [9].

The formic acid was found to produce gelatin solutions suitable for electrospinning experiments. Solutions of 7-12% (w/v) gelatin dissolved in formic acid were successfully electrospun into nanofibers with diameters in the range from 70 nm to 170 nm [10]. But, in time, the formic acid determines gelatin degradation and an important decrease of the solution viscosity, which makes the electrospinning process impossible due to beads formation [10].

The second important problem in gelatin electrospinning is the gelatin concentration of the electrospinnable solution. It was reported that gelatin solutions with gelatin concentrations higher than 12% could not be electrospun because a hardened gelatin phase developed on the edge of the needle tip, disturbing the fluid filament flow and the quality of the resulted nanofibers [3].

However, electrospinning of high concentration gelatin solutions is desirable, because the initial gelatin concentration was shown to be the most important parameter in controlling the cross-linking density and cytotoxin formation in gelatin biomedical applications [11, 12]. In the present work, we investigated the possibility to increase the gelatin concentration of electrospinnable solutions by using mixtures of formic acid (FA), acetic acid (AA) and dimethylformamide (DMF) in suitable

ratios. The optimized solvent mixture was found to extend the solution stability interval, also.

2. Experimental

2.1. Gelatin preparation

A gelatin (Type B) solution was prepared from hides of young bovines, following the method described in [13]. The gelatin solution was dried in a thermostat under air flow, obtaining gelatin solid sheets. The gelatin sheets were freeze dried (Manifold Freeze Dryer, Millrock Technology) and ground to powder (Lab Benchtop Colloid Mill, Sonic Corporation).

2.2. Gelatin solutions

The gelatin solution investigated in the present work were prepared using the above described gelatin powder, formic acid (98%, Kanto Ltd), acetic acid (66%, Sigma Aldrich), dimethylformamide (Merck) and deionised distilled water. The solutions with 27%, 30% and 33% (w/v) gelatin content, were prepared by dissolving gelatin powder in formic acid and/or the following solvent mixtures: FA:AA (4:1), FA:DMF (4:1) and FA:AA:DMF (4:0.5:0.5). The solutions were stirred at room temperature for 3h and the impurities and inhomogenities were removed using a stainless steel filter.

2.3. Viscosity measurements

The apparent viscosity, η_a, of the gelatin solutions was measured at room temperature (25±0.1°C), at 3, 6, 9, 12, 24 and 48 hours after preparation, using a rheoviscosimeter type Rheotest 2 (Prüfgerätewerk Medingen) with coaxial cylinders, following ISO 3219/1993 requirements [14]. The results were analyzed according to the power law relationship between the shear stress and the shear deformation rate.

2.4. Gelatin films

A reference film of 30% (w/v) gelatin was prepared from gelatin powder dissolved in distilled deionised water. Films were prepared from all the investigated gelatin solutions by casting on glass substrates and drying at room temperature for 24h in a thermostat, under air flow. The experiments were performed at room temperature in a room with less then 50% relative humidity.

2.5. Gelatin electrospinning

The electrospinning set-up consisted of a 10 ml syringe with stainless steel blunt needle (0.5 mm inner diameter), a home-made syringe pump, an aluminium foil as fiber collector, and a Brandenburg Alpha III Series Precision Laboratory HV Power Supply (30V-30kV). The spinning geometry was vertical, the syringe needle being placed at 14 cm above the center of the collector. The connection between the syringe and the needle was done using a 40 cm long Teflon tube of 0.8 mm inner diameter. The syringe needle was the anode, and the aluminium foil was the cathode. All the solutions were electrospun using the same electrospinning parameters: 19.5 kV dc voltage applied between needle and plate, and 3.7 μL/min solution flow rate.

2.6. Structural characterization

The morphology of the electrospun gelatin nanofibers was examined by scanning electron microscopy (SEM) using a Vega 2 Tescan (Czech Republic) microscope with Atlas Tescan software for image analysis.

The chemical structure of the gelatin films and nanofiber membranes was analyzed by Fourier-transform infrared attenuated total reflectance spectroscopy (FTIR-ATR) using a DIGILAB – SCIMITAR Series FTS 2000 spectrometer with ZnSe crystal, 750-4000 cm^{-1} range, 4 cm^{-1} resolution.

The crystallinity of the gelatin nanofibers was evaluated from wide-angle X-ray diffractograms recorded with a Philips X'Pert Pro Multipurpose X-ray Diffractometer operated at 40 kV and 40 mA.

Thermal properties of the electrospun membranes and gelatin films were analyzed by recording differential scanning calorimetry (DSC) thermograms of 10 mg samples, using a Thermal Analysis Instrument TA 2910, at a scanning rate of 10°C/min and nitrogen gas flow rate of 50 ml/min.

3. Results and discussions

3.1. Gelatin solution stability

In our experiments we noticed that acetic acid added to a gelatin-FA solution slows down the gelatin degradation process and increases the solution viscosity, while DMF decreases the viscosity and makes the solution stable for a longer time. On the basis of these results, we supposed that adding both AA and DMF to a gelatin-FA solution would produce an electrospinnable solution with improved properties. After a series of tests, we found that a FA:AA:DMF mixture with 4:0.5:0.5 ratio, provides the longest stability and electrospinnability interval (Fig. 1). The apparent viscosity dependences on time and gelatin concentration of some solutions investigated in our work are presented in Figures 1 and 2.

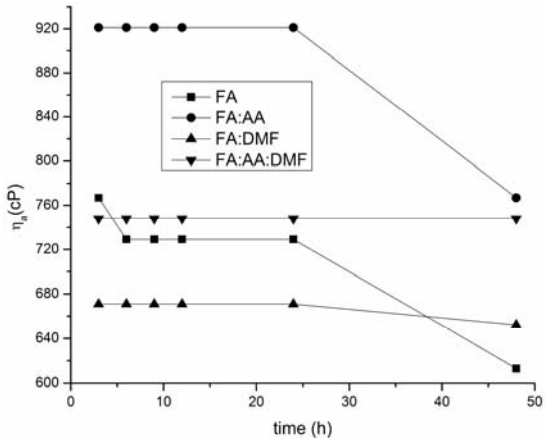


Fig. 1. Time dependence of the apparent viscosity, η_a, of gelatin solutions prepared using different solvent mixtures (the lines are just eye guides).

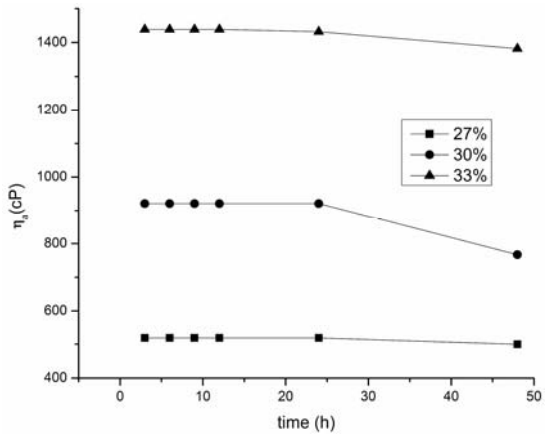
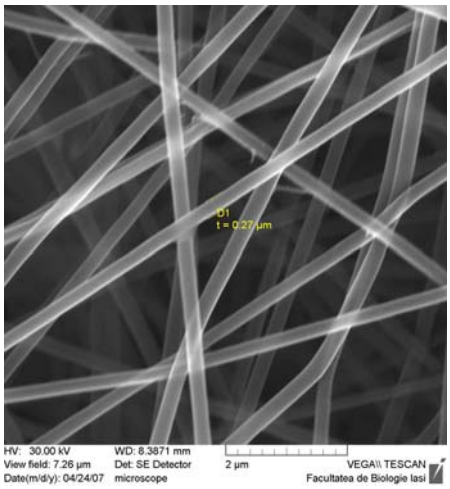


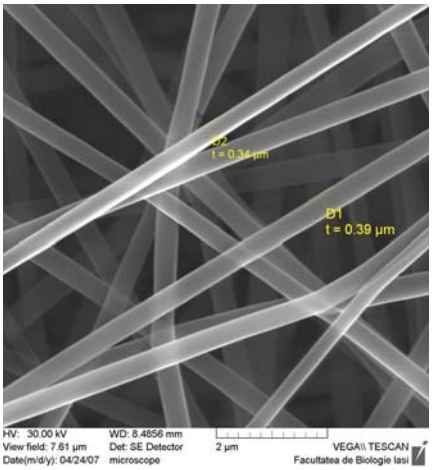
Fig. 2. *The influence of the gelatin concentration on the apparent viscosity (η_a) time variation of a gelatin-FA:AA solution (percents in the graph legend show the gelatin concentration).*

It is well known that weak acids like acetic acid, determine lyotropic solubilisation [15], while DMF which is a hydrophilic aprotic solvent, is able to bind water and decrease the surface tension of a solution. When acting together, acetic acid and DMF improve gelatin swelling by the two mechanisms: the lyotropic swelling due to the water brought by the non-ionized acid species (water bound by hydrogen bonds to non-ionisable groups), and the osmotic swelling by releasing groups involved in intermolecular interactions through hydrogen bonds [16].

The increased stability of the gelatin solutions prepared with FA:AA:DMF solvent mixture is probably due to the formation of temporary hydrogen bonds and helix-type association of gelatin molecules that keep the solution structurally stable. The formation of gelatin molecule associations intensifies when the gelatin concentration increases, as proved by the viscosity data presented in Fig. 2 for gelatin-FA:AA solutions.



(a)



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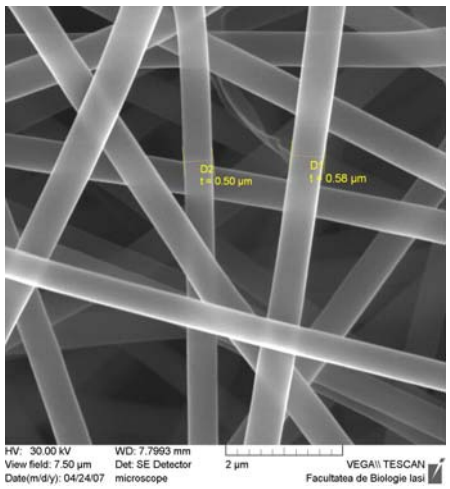


(c)

Fig. 3. Scanning electronic microscopy photographs of gelatin nanofibers electrospun from gelatin dissolved in formic acid - acetic acid mixture, FA:AA = 4:1 ratio. Gelatin content (w/v): a) 27%, b) 30%, c) 33%.

3.2. Gelatin nanofiber morphology

In our electrospinning experiments performed at room temperature, continuous nanofibers were successfully electrospun from all the solutions mentioned above. The nanofibers morphology and the average diameter of the nanofibers were determined from SEM images taken at ten different locations on the surface of each investigated membrane.

The gelatin electrospun membranes consisted of uniform nanofibers deposited randomly on the collector surface, without connecting at intersection places, arranged in a 3D structure with pores of about 2 micrometers.

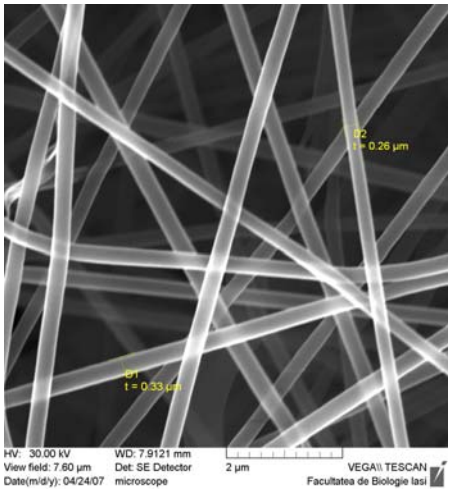
Using the same electrospinning parameters mentioned above (i.e. 14 cm, 19.5 kV, 3.7 μL/min), the gelatin-FA:AA:DMF solutions with 27% and 30% (w/v) gelatin, allowed the deposition of sufficiently thick nanofiber membranes in only few minutes (Fig. 3).

In case of solutions with 33% (w/v) gelatin, we observed the same phenomenon reported by other authors [3]. Periodically, a cloggy particle developed on the edge of the needle tip. When the particle diameter reached about 1 mm, it was detached by the incoming jet and carried towards the collector. This phenomenon was observed for all the solutions of gelatin-FA:AA and gelatin-FA:DMF type. However, because the development of a hardened gelatin particle took about 15 minutes, there was enough time to collect nanofiber membranes without defects for all the gelatin-FA:AA solutions. Increasing the gelatin content of a FA:AA type solution from 27% to 33% (w/v), determined a rapid increase of the nanofibers average diameter from 260 nm to more than double.

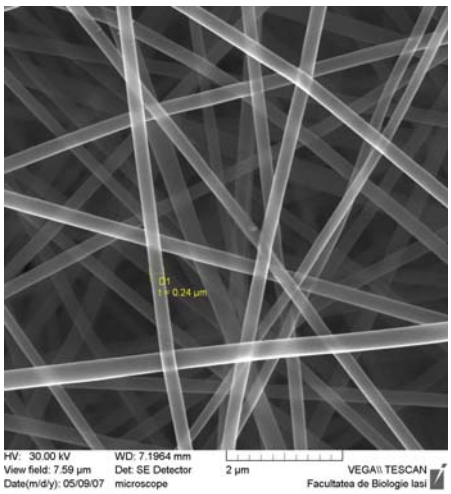
The SEM images presented in Fig. 4 illustrate the effect of various solvent mixtures on the morphology of gelatin nanofibers prepared from solutions of 30% (w/v) gelatin. Figure 4(a) shows nanofibers with 300 nm average diameter, prepared from a gelatin-FA solution. After adding DMF, as known from the viscosity measurements, the apparent viscosity of the solution decreased and the nanofibers became thinner, with an average diameter of 240 nm (Fig. 4(b)). When all the solvents acted together in a gelatin-FA:AA:DMF solution, the average diameter of nanofibers increased at 420 nm, in agreement with the increase of the apparent viscosity due to the acetic acid action (Fig. 1). The nanofiber average diameters observed in our investigation are presented in Table 1.

Table 1. Gelatin nanofibers average diameter (d_{av}) as a function of solvent type and gelatin concentration (c_G) of the electrospun solution.

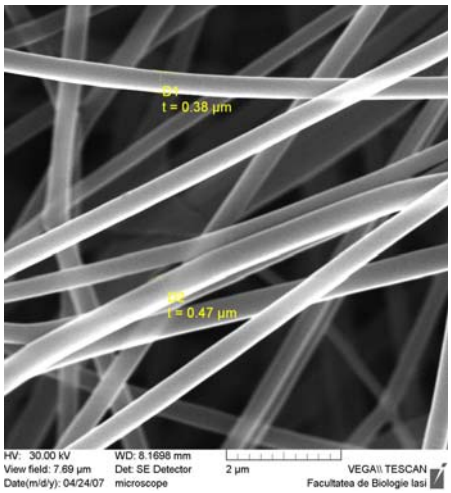
Solvent	c_G (% w)	d_{av}(nm)
FA	30	300
FA:AA	27	260
FA:AA	30	360
FA:AA	33	540
FA:DMF	30	240
FA:AA:DMF	30	420



(a)



(b)

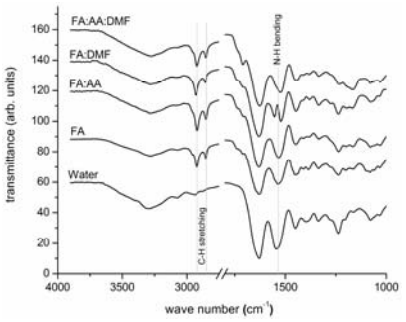


(c)

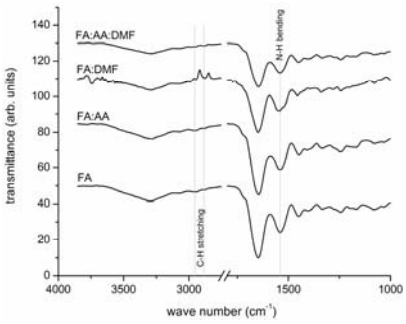
Fig. 4. *Scanning electronic microscopy images of gelatin nanofibers prepared by electrospinning of gelatin dissolved in: a) FA, b) FA:DMF, c) FA:AA:DMF. The gelatin concentration of all solutions: 30% (w/v).*

3.3. Structural characterization by FTIR, DSC and XRD

Fig. 5(a) shows important differences between the FTIR spectra of gelatin films and those of gelatin nanofiber membranes. The gelatin films prepared with solvent mixtures have peaks at 2922 cm^{-1} and 2848 cm^{-1} (C-H stretching), due to reorientation of gelatin molecules interconnected by CH_2 peptide bonds [17]. These bonds are promoted by the formic acid and do not exist in films prepared from aqueous solutions. The structuring process of gelatin films is favoured by the slow evaporation of solvents. On the contrary, in gelatin nanofibers the evaporation of solvents is very fast and the molecular reorientation is difficult. As a consequence, the two C-H stretching peaks are absent in the FTIR spectra of gelatin nanofibers presented in Fig. 5(b).



(a)



(b)

Fig. 5. FTIR spectra of gelatin films (a), and electrospun gelatin membranes (b), prepared using various solvent mixtures. Gelatin concentration of all solutions: 30% (w). The two small peaks on the FA:DMF trace near the C-H stretching band are due to the aluminium foil substrate.

The FTIR spectra of gelatin films prepared with FA:DMF mixture show that the N-H bending peak at 1530 cm^{-1} (Amide II) splits into two peaks located at 1560 cm^{-1} and 1515 cm^{-1}. This splitting is probably due to the DMF action on the peptide group, allowing the formation of -CNH links while blocking the formation of carboxyl (COOH) and amide (-CONH) links. The predominant -CNH links determined the formation of a more ordered structure of gelatin-FA:DMF films during drying, as confirmed by the DSC trace of the gelatin-FA:DMF film characterized by a single endothermic peak (Fig. 6).

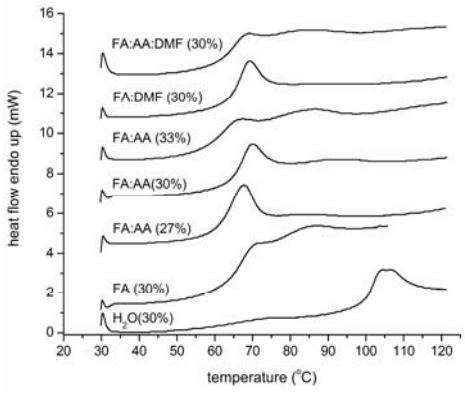


Fig. 6. *DSC thermograms of gelatin films prepared using various solvent mixtures. The percent in parenthesis shows the gelatin concentration.*

The FTIR spectra of the nanofiber membranes (Fig. 5(b)) look almost identical, irrespective of the solvent mixture used. This is a good result, proving that there is no need for further treatments to remove undesired structures in the electrospun gelatin nanofiber mats. The identical structure of the electrospun gelatin nanofibers is a result of the intense electric field action and of the very fast evaporation of solvents from the solution filaments. The rapid elongation of the solution jet stretched by electrical forces breaks the interchain bonds, while the fast evaporation of solvents freezes the disordered state leading to the formation of amorphous nanofibers.

The amorphous structure of the electrospun gelatin nanofibers is confirmed by the X-ray diffractograms presented in Fig. 7. The broad peaks on some of the X-ray diffractograms are coming from the different fixtures used to hold the investigated samples. We used various methods in searching for some preferential molecular orientation in the gelatin nanofibers, but all the membranes appeared completely amorphous.

The different molecular ordering processes promoted in gelatin films by the various solvent mixtures are clearly visible on the DSC thermograms (Fig.6). The gelatin film prepared form an aqueous solution shows a main endothermic peak at 125°C, due to the glass transition superposed with water evaporation. On the contrary, almost all the gelatin films prepared with solvent mixtures show two endothermic peaks, around 68°C and 88°C. The difference is made, the same as in case of FTIR spectra, by the film prepared with the FA:DMF mixture which shows only one endothermic peak at 69.5°C. The absence of the second endothermic peak is a result of the same molecular interactions that caused Amide II peak splitting observed on the FTIR spectra (Fig. 5(a)), and determined the largest decrease of the viscosity noticed among all the solutions investigated (Fig. 1).

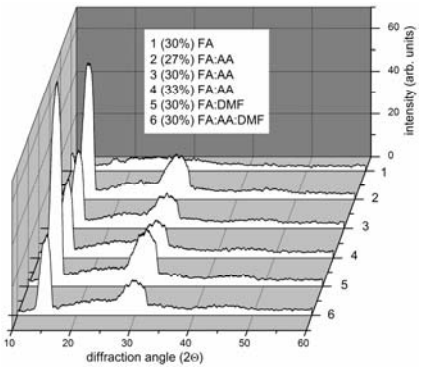


Fig. 7. X-ray diffractograms of gelatin nanofiber membranes, showing complete amorphous structure irrespective of the solvent mixture used. The percents on the graph legend refer to the gelatin concentration. The wide peaks at 15° and 28° are coming from the sample holder.

The second endothermic peak showed by the DSC records of gelatin films presented in Fig. 6, appears to be very sensitive to the gelatin concentration. The films prepared from gelatin-FA:AA solutions of 27%, 30% and 33% (w/v) gelatin, show clearly that a higher gelatin concentration increases the surface of the second endothermic peak. This observed increase is probably a consequence of the competition between acetic acid molecules and gelatin molecules to occupy the valence sites on CO and NH groups of the peptide bonds, resulting in different molecular reorientation processes during films drying [18].

4. Conclusions

Gelatin nanofibers were successfully prepared by the electrospinning of gelatin solutions with gelatin concentrations of 27% and 30% (w/v). The membranes consisted of uniform nanofibers randomly oriented, forming 3D structures with pores of about 2 micrometers. Increasing the gelatin concentration caused the increase of the gelatin nanofiber diameter from 200 nm to 580 nm.

Various mixtures of formic acid, acetic acid and dimethylformamide were used in searching for an optimized gelatin solution with maximum gelatin concentration and longest stability. It was found that a solvent mixture of FA:AA:DMF with 4:0.5:0.5 ratio gave the best results, the solution remaining stable and spinnable for more than 48 hours. The highest gelatin concentration of a FA:AA:DMF type solution allowing a good quality and stable electrospinning process was, in our experiments, 30% (w/v).

Electrospinning of gelatin solutions prepared with a FA:AA:DMF solvent mixture does not require adjustment of the electrospinning parameters when the gelatin concentration varies in the range 27-30% (w/v), nanofiber membranes being successfully prepared from these solutions using the same electrospinning parameters.

Electrospinning of high concentration gelatin FA:AA:DMF solutions allows a fast deposition of gelatin non-woven mats of enough thickness and mechanical strength required in biomedical applications.

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