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Electrospinning of gelatin/chitin composite nanofibers

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Gelatin/chitin nanofibers were prepared by electrospinning. Various solutions were obtained by mixing a 27 % (w/v) gelatin in formic acid solution with crab shell chitin dissolved in a mixture of trichloroacetic acid and formic acid in 1:4 volume ratios. Obtaining electrospinnable gelatin/chitin solutions required the decrease of chitin molecular mass. Two types of depolymerisation processes were tested: microwave irradiation and ultrasonic treatment. By variation of irradiation parameters, an optimum was found with the microwave reactor working in temperature control mode. The chemical and physical structure of the gelatin/chitin nanofibers were investigated by scanning electron microscopy and infrared spectroscopy.

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1. Introduction

In the last ten years, there was an increasing interest in tissue engineering for the development of scaffolds using biodegradable and biocompatible natural polymers.

In natural tissues, the wound healing process involves cells attachment and proliferation inside the extracellular matrix, a three-dimensional network structure made of collagen multifibrils and proteoglycans, with diameters in the range from 50 nm to 500 nm [1].

A good scaffold should mimic the structure and biological function of the proteins in the natural extracellular matrix, in order to provide mechanical support for cell attachment and facilitate all the necessary cell activities. The scaffold material and its texture were reported to have an important influence on the shape and function of the regrowth tissue [2-4].

The above mentioned requirements are very well fulfilled by an electrospun nonwoven membrane consisting of nanofibers arranged in a geometry similar to the collagen structure of the natural extracellular matrix. When sugar- and amino- acid residues are included in the structure of these nanofibers, they are able to stimulate physiological responses similar to those triggered by the proteins and growth factors acting in the human extracellular matrix during tissue regeneration and restructuration.

In the present work, we investigated a blend of chitin and gelatin as a candidate material for wound dressings. The chitin monomer unit, N-acetylglucosamine, occurs also in hyaluronic acid known as an extracellular macromolecule important in the wound repair processes. Chitin has also structural characteristics similar to glycosaminoglycans such as chondroitin sulfates which contribute to control the tensile strength of tissues [5]. Gelatin is basically denaturated collagen and offers all the surface structural elements needed for cell attachment and migration.

The chitin existing in the gelatin/chitin nanofibers applied on the wound surface could stimulate macrophages for secretion of lysozyme and human chitinase, enabling in this way the immune system to prevent infection by chitin-based pathogens [6]. Besides this, chitin is hydrolyzed to chitooligomers which stimulate other macrophage functions like collagen deposition, nitric oxide and tumor necrosis factor production [7]. Therefore, we believe that nanofibers made of a gelatin/chitin blends may possess all the important characteristics needed for promoting rapid dermal regeneration and accelerating wound healing.

2. Experimental

2.1. Materials

The gelatin/chitin blends investigated in this work were prepared from crab shell chitin (Sigma-Aldrich, practical grade) and gelatin (Fisher, HealthCare), used as received. As solvent for chitin, a mixture of trichloroacetic acid (Scharlau Chemie, reagent grade) and formic acid (Scharlau Chemie, 98-100%) in volumetric ratio 1:4 was used. Gelatin was dissolved in formic acid, 27% (w/v), by stirring at room temperature.

2.2 Chitin depolymerisation

Chitin solutions were prepared by dispersing the chitin powder in suitable quantities of solvent (trichloroacetic and formic acids), followed by ultrasonic or microwave irradiation. Ultrasonic treatments were performed using a Sonoplus HD 2200 (Bandelin Electronic) ultrasonic homogenizer working at 20 kHz, able to provide sonic power up to 200W, to solution volumes in the range 1-200 ml. For microwave irradiation, a STAR-2 (CHEM Corp.) microwave reactor was used, working at 2.45 GHz frequency and 800 W maximum power.

2.3 Structural characterization

The apparent viscosity, η_a, of chitin solutions in alkali measured at room temperature (25±0.1°C), using a Nahita Rotary Viscometer 801, observing ISO 3219/1993 requirements [8].

The intrinsic viscosity η of the chitin solution samples dissolved in alkali (2.77 M NaOH) was measured with an Ubbelohde U-tube viscometer using the relation:

$$\eta = t_{ch} / t_s$$

where t_{ch} and t_s are the flowing intervals of the chitin solution and the solvent.

The molecular mass of chitin in all the investigated solutions was computed using the correlation between intrinsic viscosity and molecular weight given by the Mark-Houwink-Sakurada-equation:

$$\eta = KM_W^a$$

where K and the exponent a are temperature dependent parameters for a given polysaccharide solvent system [9, 10]. The exponent a is a polymer conformation parameter that decreases with increasing molecular compactness. In our experiments, the molecular weight of the chitin samples were computed using K=0.10 and a=0.68 [11]. The viscosity measurements on as received chitin gave η=935 ml/g and M_W=1125 kg/mol.

The structural changes of chitin solutions after ultrasonic or microwave treatments were investigated by infrared spectroscopy, using a Bruker Tensor 27 FT-IR Spectrometer with Platinum ATR single reflection diamond ATR sampling module (Bruker Optics), working from 4000 to 400 cm^{-1}.

The chemical structure of the gelatin/chitin nanofiber membranes was analyzed by FTIR-ATR using a DIGILAB – SCIMITAR Series FTS 2000 spectrometer with ZnSe crystal, working in the range 4000-750 cm^{-1} with 4 cm^{-1} resolution.

2.4 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 HV Power Supply (30V-30kV). A vertical geometry was selected in which the syringe needle was placed at 12 cm above the collector center. The syringe and the blunt needle were connected by a 40 cm long Teflon tube of 0.5 mm inner diameter. The syringe needle acted as anode, and the aluminium foil was the cathode. Between needle and collector dc voltages in the range 10-14.5 kV have been applied. The solutions were electrospun at solution flow rates between 2.4 and 3.7 μL/min.

3. Results and discussions

The most important step in electrospinning a polymer is the preparation of a suitable solution. The electrospinning process stability and the nanofiber morphology are influenced by the physical properties of this solution. An electrically stretched polymer solution jet will form a fiber only if a balance between the surface tension and the viscosity of the solution is acquired. The solution surface tension is very much influenced by the surface tension of the solvent. The solution viscosity depends on the molecular mass of the polymer, which is proportional with the macromolecules length. When polymer molecules are too short, there is insufficient entanglement between the molecular chains and the solvent surface tension becomes dominant, giving rise to beads along the fiber. If polymer molecules are too long, i e. molecular mass too high, the solution viscosity is high and jet stretching by electrical forces becomes difficult or even impossible [12].

The chitin extracted from crab shells (α-chitin) is characterized by a strong three-dimensional hydrogen bond network of antiparallel unit cell (*N*-acetylglucosamine) stacks, which makes swelling and dissolution processes very difficult. As extracted α-chitin from crab shells has a high molecular mass of 1125 kg/mol, as derived from viscosity measurements.

The poor solubility of chitin is a result of the close packing of chains and its strong intra- and intermolecular bonds between the hydroxyl and acetamide groups [13]. The inability of α-chitin to swell upon soaking in water is explained by the extensive intermolecular hydrogen bonding [14].

Under these circumstances, preparation of an electrospinnable solution requires a supplementary energy that could help the solvent to tear apart and break the long molecular chains. In our investigation, two possibilities of delivering a supplementary energy to the solvent have been tested, ultrasound and microwave (MW) irradiation.

3.1. Chitin depolymerisation by ultrasonic treatment

A first set of experiments was aimed to prepare solution with 8.00% chitin, dissolved in the above mentioned mixture of trichloroacetic acid and formic acid. A set of equal quantities of solution were prepared by dispersing chitin powder in the solvent mixture. These mixtures were subsequently subjected to ultrasonic treatment at room temperature, varying the ultrasonic

power between 50W and 200W, and the treatment duration from 15 min to 2 hours. The ultrasonic treatment intervals and the resulted chitin molar mass are presented in Table 1.

Table 1. Ultrasonic treatment parameters and resulting molar mass of a 8% (w/v) chitin solution in 1:4 trichloroacetic acid and formic acid.

Sample	Time (min)	M_w (kg/mol)
U1	15	1100
U2	30	1020
U3	60	970
U4	120	940

The solution U4, made of 8% (w/v) chitin, prepared using an ultrasonic treatment of maximum power and duration, i.e. 200W and 2h, at room temperature, formed electrospun nanofibers with a very wide range of diameters (Fig. 1), demonstrating an unstable electrospinning process due to the high viscosity and molecular mass of chitin. The high viscosity and concentration of chitin caused a frequent breaking of the polymer jet and the fiber diameter resulted very nonuniform. This result suggests that increasing the power and duration of the ultrasonic treatment does not lead to an uniform chain fragmentation. The solution remains viscous even after long treatment intervals, suggesting that the molecular chains are able to rapidly restore the interchain bonds broken by the ultrasonic energy. In order to decrease the viscosity an increase the efficiency of the ultrasonic treatment, we decreased the chitin content of the blend.

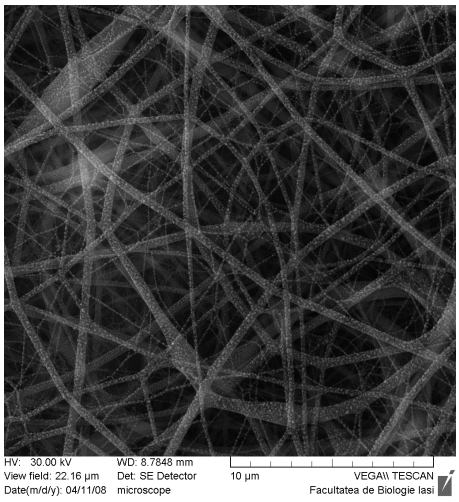


Fig. 1. SEM image of nanofibers prepared by electrospinning a solution of 8.00% (w/v) chitin in 1:4 (v/v) trichloroacetic acid and formic acid.

Decreasing the chitin concentration to 1.33% (w/v), the 2h ultrasonic treatment at 200W became more effective (M_W=850kg/mol) and the electrospinning of the resulting solution lead to a slightly more stable electrospinning process, with a narrower distribution of fiber diameters. However, because the molecular length remained high and the molecules concentration low, the solvent accumulate sometimes under the action of surface tension and formed the beads seen of Fig. 2. A higher quantity of solvent was more difficult to evaporate, and the fibers in Fig. 2 manifested the tendency to bind at the connection points.

3.2. Chitin depolymerisation by microwave treatment

The conclusion of the above described treatment is that supplying by ultrasound a supplementary energy to the solvent molecule aiming to make them more effective in breaking the hydrogen and the β-glycosidic bonds for cutting the chitin chains in shorter pieces, is not working. The ultrasonic energy involves only mechanical aspects of molecular movement. Ultrasound energy increases the brownian movement energy of solvent molecules, but this energy is dissipated homogenously on the chitin chains length.

In order to obtain an efficient molecular chain cutting, the supplementary energy should be focused on the bonds of interest, making them more susceptible for breaking. Microwave irradiation appears to be a more efficient form of stimulation, because it acts by stimulation the vibration of the bonds and enhances the electrical attraction between these activated bonds and the charged groups of the solvent molecules.

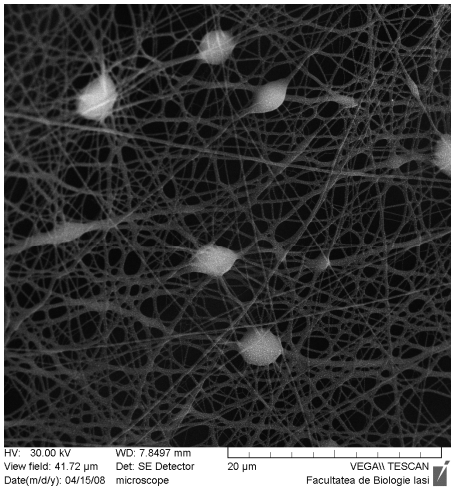


Fig. 2. SEM image of nanofibers prepared by electrospinning a solution of 1.33% (w/v) chitin in 1:4 (v/v) trichloroacetic acid and formic acid.

A second set of experiments were bound to finding the microwave treatment optimum parameters. Two sets of mixtures chitin powder/solvent were subjected to microwave treatments.

The CHEM STAR-2 microwave reactor has two possible modes of working. The first mode, named *Power Control* (PC), means that a percent of the full power (800 W) acts on the irradiated sample, while the sample temperature evoluates freely. In the second mode, called *Temperature Control* (TC), the reactor acts with pulses of

full power, keeping the sample temperature at a constant value. In our experiments, a separate set of test have been performed for each working mode of the STAR-2microwave reactor.

In the first set of MW irradiation experiments, the reactor was working in the PC mode, following the parameters presented in Table 2. The investigated samples were appropriate quantities of chitin powder/solvent mixture, placed in standard Pyrex glass 10 ml tubes.

Table 2. Microwave treatment parameters, Power Control mode (PC), duration 3 minutes.

Sample	Power (%)	T_{max} (^oC)	M_w (kg/mol)
PC1	15	85	22.06
PC2	10	80	22.79
PC3	6	76	19.56
PC4	5	70	25.85

The structural modifications of the chitin solutions after each treatment were investigated by FTIR spectroscopy. The recorded spectra of the MW treated chitin solutions in PC mode are shown in Fig. 3.

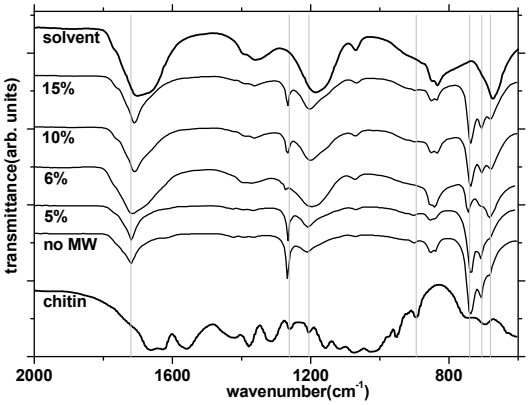


Fig. 3. FTIR spectra of chitin dissolved in a mixture of trichloroacetic acid and formic acid, subjected to microwave treatment in power control mode.

A second set of MW irradiation experiments were performed on chitin/solvent mixtures irradiated with the reactor working in TC mode, keeping the temperature at 70^{o}C and changing the treatment interval. The irradiation parameters are given in Table 3, and the FTIR spectra of the corresponding treated chitin solutions are shown in Fig. 4.

Table 3. Microwave treatment parameters, Temperature Control mode, T_{max} = 70^{o}C .

Sample	Power (%)	Time (min)	M_w (kg/mol)
TC1	100	15	19,56
TC2	100	5	26,03
TC3	100	3	29,46
TC4	100	1	36,03

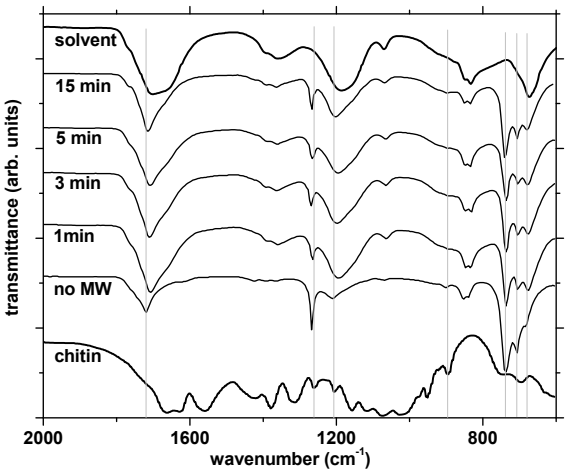


Fig. 4. FTIR spectra of solution of chitin dissolved in trichloroacetic acid and formic acid, subjected to microwave treatment in Temperature Control mode, temperature T_{max}=70^{o}C.

Samples of chitin solutions form PC-series and TC-series were mixed with 27% solution of gelatin dissolved in formic acid and tested for electrospinning. The electrospinning results showed that all the gelatin/chitin solution prepared with PC or TC microwave irradiated samples allowed stable electrospinning processes, but the nanofibers morphology presented some important differences depending on the microwave treatment type applied.

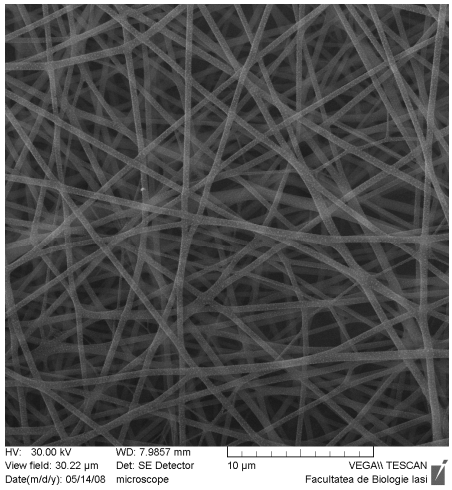


Fig. 5. SEM image of nanofibers prepared by electrospinning a solution of 1.33% (w/v) chitin in 1:4 (v/v) trichloroacetic acid and formic acid, treated by microwave irradiation in Power Control mode, 6% of maximum power (800W), for 3 minutes.

Molecular mass measurements and FTIR spectra of the microwave treated chitin solutions, together with SEM images of the nanofibers electrospun from gelatin/chitin blends prepared using these chitin solutions, proved that chitin subjected to microwave irradiation undergoes both physical and chemical transformations.

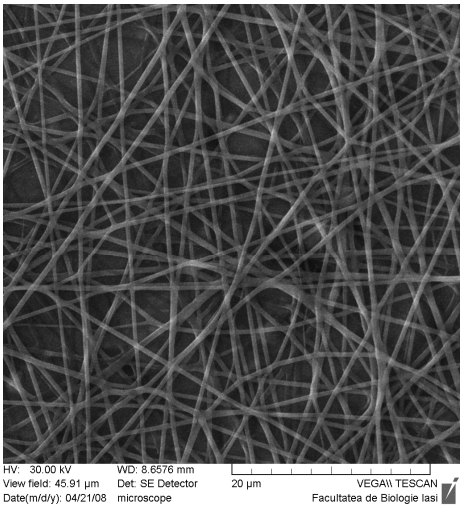
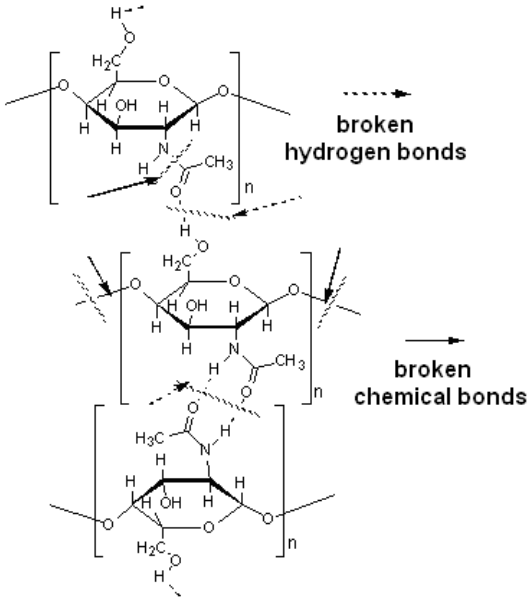


Fig. 5. SEM image of nanofibers prepared by electrospinning a solution of 1.33% (w/v) chitin in 1:4 (v/v) trichloroacetic acid and formic acid, treated 5 min by microwave irradiation in Temperature Control mode.

The physical transformations involve breaking of hydrogen bonds between macromolecular chains, increasing the distance between them and creating the possibility of breaking a chain into shorter pieces. The chemical transformations could consist of breaking etheric (positions 1-4 of the pyranosic ring) and estheric (amine group) bonds.



The shape of solution temperature time dependence in case of TC mode treatments, show that after a first interval of about 30 seconds, the chitin solution begins to absorb very fast the microwave energy, and after other 30 seconds the degradation of chitin ends, the temperature reaching a flat level.

The molecular mass of as received crab shell chitin, 1150±30 kg/mol, after MW treatment in solvent drops to 22±4 kg/mol, as proved by viscosity measurements. This result suggests that the MW treatment broke a macromolecular chitin chain in about 50 shorter pieces of close lengths. In this way, the resulting chitin keeps its physical and chemical macroscopic properties, but the solution properties change dramatically as a result of the new chain packing mechanisms possible between the shorter chains.

The as received chitin has long chains which tend to align parallel, giving rise to a long range order. In MW treated chitin solutions, the shorter chains are free to pack in a more diverse and flexible ways, as shown qualitatively in Fig. 6.

The macromolecular packing mechanism presented in Fig. 6b is supported by the FTIR spectra of the MW treated chitin solutions, which show changes in absorption bands of –NH, =C=O, -CH and CH_2 groups, as will be detailed in the following.

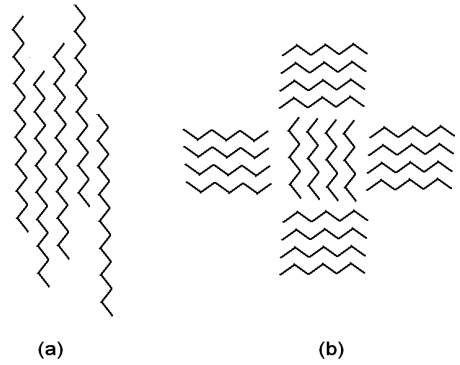


Fig. 6. Macromolecular chains packing in crab shell chitin solutions: (a) as received (long chains), and (b) after microwave treatment (short chains).

The changes of hydrogen bonds and hydrogen-bonded groups can be observed on FTIR spectra [14]. In the FTIR spectra shown in Figs. 3 and 4, the peaks were assigned as follows: 1262 cm^{-1} to amide III (δ_{NH}), 705 and 680 cm^{-1} to amide V (γ_{NH}), 895 cm^{-1} to γ_{CH} and 740 cm^{-1} to ρ_{CH2} [16].

The deformation bands of the –CH group (γ_{CH}) show a decreased intensity after the microwave treatment of the chitin solution. This behaviour could appear because the anomeric carbon C_1 has a C_1-H oxidic bond, i.e. a $\beta_{1\text{-}4}$ bound with the next ring, possible to appear only in a packing system as shown in Fig. 6b.

The absorption band at 1750-1650 cm^{-1}, assigned to C=O stretching (amide I) of the MW treated chitin solutions appears broader as compared to the similar bands of the as received chitin, suggesting that a larger number of shorter chains interact by hydrogen and van der Waals bonds. Moreover, the C=O band of the as received chitin is centered at aprox. 1720 cm^{-1}, while the similar band of all MW treated chitin solutions is shifted with 10-40 cm^{-1} towards lower frequencies, suggesting an increased number of hydrogen bonds appeared between shorter chains (Fig. 6b).

The NH amide group has absorption bands due to various types of deformations [17]. In case of MW treated chitin solutions, the intensity of NH bands at 1260 cm^{-1}

and 705 cm^{-1} slightly decreases and shifts to lower frequencies, suggesting a (small) partial deacetylation of the NH amide group.

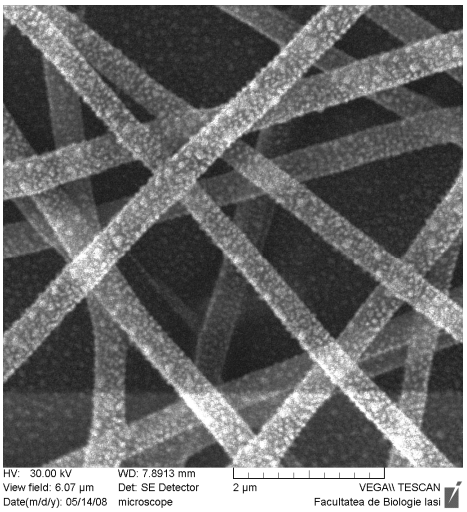


Fig. 7. SEM image of gelatin/chitin nanofibers prepared by electrospinning a blend of 1.33% (w/v) chitin in 27% (w/v) gelatin/formic acid. The chitin solution was irradiated with microwave in Power Control mode, 15% of the total power (800W) for 3 minutes. The excess power lead to the formation of low molecular fragments accumulated on fibers surface during solvent evaporation.

Another chemical transformation that could lead to the observed changes in the NH band is the interaction of the NH group with an electrophile group of a chain fragment like CH_3-COOH. Low molecular fragments could explain the presence of some particles observed on the SEM images of the gelatin/chitin nanofibers prepared with non-optimal MW treated chitin solutions (Fig. 7).

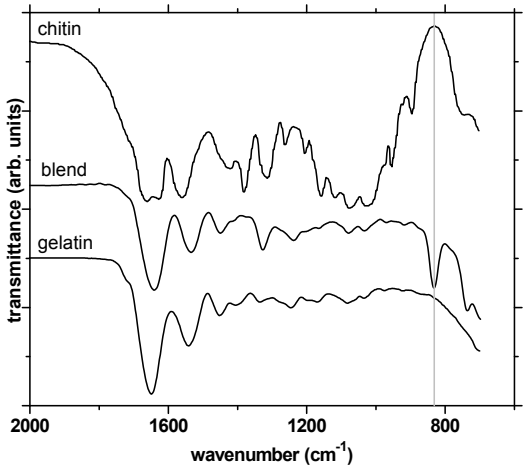


Fig. 8. FTIR-ATR spectra of gelatin, chitin and gelatin/chitin nanofibers prepared using a chitin solution irradiated with MW in the TC mode for 5 minutes. The peak at 840 cm^{-1} suggests a chemical interaction between gelatin and chitin.

In Fig. 8 is presented a FTIR-ATR spectrum of a gelatin/chitin nanofibers membrane prepared in the optimal conditions found in the present study, i.e. the chitin solution MW irradiated in TC mode for 5 minutes before mixing with the 27% gelatin/formic acid solution. As one can see in Fig. 8, the absorption peak at 840 cm^{-1} does not belong to gelatin or chitin in their pure forms. This peak suggests a possible chemical bond between gelatin and chitin chains in the nanofibers structure. The mechanism responsible for this absorption is currently under investigation.

The other bands of the gelatin/chitin nanofibers are basically the bands of gelatin, some of them having increased intensities as a result of interchain interactions with chitin.

4. Conclusions

In the present work, gelatin/chitin nanofibers with 1.33% (w/w) chitin have successfully prepared by electrospinning.

In order to obtain an electrospinnable solution, the molecular mass of the as received crab shell chitin was decreased (chitin depolymerisation). Two possible depolymerisation methods have been tested, ultrasound and microwave irradiation.

It was found that microwave irradiation gives the best results, with the reactor working in temperature control mode. The optimum solution was prepared by irradiating 10 ml of gelatin/solvent mixture, using pulses of 800W microwave power for 5 minutes, at 70°C. The gelatin/chitin blend prepared using this chitin solution gave the best quality of the electrospun nanofibers.

All the other gelatin/chitin blends prepared with microwave irradiated chitin solutions were electrospinnable. The main difference from the optimum nanofibers was the presence of low molecular fragments accumulated as a "dust" on the nanofibers surface. The low molecular fragments appeared as a consequence of excessive microwave power or irradiation time.

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