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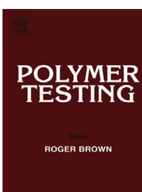

Material behaviour

Thermal and hydrolytic degradation of electrospun fish gelatin membranes

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ABSTRACT

The thermal and hydrolytic degradation of electrospun gelatin membranes cross-linked with glutaraldehyde in vapor phase has been studied. *In vitro* degradation of gelatin membranes was evaluated in phosphate buffer saline solution at 37 °C. After 15 days under these conditions, a weight loss of 68% was observed, attributed to solvation and depolymerization of the main polymeric chains. Thermal degradation kinetics of the gelatin raw material and as-spun electrospun membranes showed that the electrospinning processing conditions do not influence polymer degradation. However, for cross-linked samples a decrease in the activation energy was observed, associated with the effect of glutaraldehyde cross-linking reaction in the inter- and intra-molecular hydrogen bonds of the protein. It is also shown that the electrospinning process does not affect the formation of the helical structure of gelatin chains.

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1. Introduction

Gelatin is a biodegradable, biocompatible, non-toxic and non-carcinogenic biopolymer [1]. It is typically obtained through partial denaturation of collagen and accounts for 30% of the total animal protein in all animals. Collagen comprises all 20 amino acids in its three α-chains, which are stabilized and interlaced by hydrogen bonds into a triple helix that rotates clockwise [2–4]. Collagen may be partially degraded using two distinct pre-treatments, the acid and the alkali, resulting in type-A and type-B gelatin, respectively [3,5]. Gelatin possesses some drawbacks regarding long term applications, such as drug delivery systems [6] or smart packaging [4,7], because the protein dissolves quickly in an aqueous environment. To overcome this limitation, cross-linking of gelatin is necessary.

Electrospinning allows the production of flexible and highly porous nanofiber structures by applying a high electric field to a droplet of polymer solution or melt [8,9]. Although gelatin has been successfully electrospun into fibers, the preparation of electrospun membranes raises some critical issues, such as the use of highly toxic solvents. Electrospun gelatin membranes can be obtained by the dissolution of the polymer in acetic acid/formic acid mixtures [10], 1,1,1,3,3,3-hexafluoro-2-propanol [11], 2,2,2-trifluoroethanol [12] and with acetic acid and ethyl acetate aqueous solutions [13].

Cross-linking of gelatin fiber membranes is a necessary step to increase its stability in aqueous environments. This can be achieved either using physical methods, such as

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dehydrothermal treatment and ultraviolet or gamma irradiation, or chemical methods which exploit a large number of chemical agents to modify gelatin functional side groups [14].

Gelatin chemical cross-linking can be achieved by glutaraldehyde (GA) [15,16], genipin [17,18] and EDC/N-hydroxysuccinimide (NHS) [19] in order to maintain morphological integrity of the as-spun membranes. GA is one of the most widely cross-linking agents used in polymeric materials from natural origin, such as gelatin [12] or chitosan [20]. It has been reported that cross-linking of gelatin microspheres with GA takes place more rapidly and is more efficient than with genipin. A maximum cross-linking degree of approximately 90% is achieved in less than 4 h of exposure to GA, whereas just around 60% of cross-linking was achieved after 72 h of exposure to genipin [21,22].

In vitro degradation of gelatin is usually performed in a PBS collagenase solution at 37 °C. Rosellini et al. [23] studied the effect of the presence of collagenase in the phosphate buffer saline (PBS) solution during *in vitro* degradation of gelatin scaffolds cross-linked with GA, and found that a mainly hydrolytic degradation process occurs due to solvation and depolymerization of the polymeric chains [24].

Studies on the thermal properties of gelatin showed that it exhibits three main thermal degradation steps, the first being attributed to water desorption, and the others associated with protein degradation [25,26]. Gelatin thermal degradation kinetics has an activation energy (E_{act}) ranging between 175 and 275 kJ.mol^{-1}, the values increasing with the protein's molecular weight [25,27].

In the present study, electrospun gelatin membranes were prepared by electrospinning and cross-linked with GA in vapor phase in order to improve the fiber membranes stability in a moisture environment. Hydrolytic degradation was evaluated in PBS solution at 37 °C. Moreover, thermal degradation before and after GA crosslinking was studied. The main goal is to evaluate the potential of fish gelatin for biomedical applications.

2. Experimental

Electrospun *membranes preparation*: Fish gelatin was purchased from Sigma–Aldrich and dissolved in a blend of N,N-dimethylformamide (DMF, from *Merck*) and formic acid (FA, from Sigma–Aldrich) (40/60 vol/vol) to achieve a gelatin concentration of 30 wt% of the final solution. Fish gelatin was dissolved by stirring at 50 °C [28]. The polymer solution was then placed in a commercial plastic syringe (10 mL) fitted with a steel needle with 500 μm inner diameter. Electrospinning was conducted at 1.5 kV.cm^{-1} with a high voltage power supply from *Glassman* (model PS/FC30P04). A syringe pump (from *Syringepump*) was used to feed the solutions into the needle tip at a rate of 0.2 mL.h^{-1}, and the electrospun fibers were collected in a grounded collector.

Cross-linking: Samples were placed in a vapor chamber and then collected at different times, between 2 and 48 h, with 20 mL of glutaraldehyde (GA, 50% water, Panreac), which was vaporized at room temperature, placed at the bottom of the chamber.

Hydrolytic degradation: In vitro degradation of cross-linked gelatin electrospun membranes was carried out in PBS solution. The membrane samples were cut into squares of 25 × 25 mm^2 (triplicate samples were used for statistical purposes), immersed in 15 mL of PBS (pH 7.4; 0.8 g NaCl; 0.2 g KCl; 1.44 g $Na_2HPO_4.2H_2O$ and 0.2 g KH_2PO_4 dissolved in 1 L of distilled water) and incubated in an air circulation oven (*HERAEUS Vacuotherm*) at 37 °C for 15 days. The pH of the PBS solution was measured periodically and PBS was renewed every 72 h. After specific periods of time, a membrane was removed from the PBS, washed with ultrapure water and dried in a vacuum oven (*JP SELECTA Vacuotherm*) at room temperature until constant mass was reached. Samples were weighed before and after degradation in an electronic quartz microbalance (M5P from Sartorius) with a resolution ≤ 0.001 mg. The extent of hydrolytic degradation (W_L) was calculated by:

$$W_L = \left(1 - \frac{m_s}{m_0}\right) \quad (1)$$

where, m_s is the sample mass after an incubation period and m_0 is the initial sample mass.

Characterization: Electrospun fibers were coated with a thin gold layer using a sputter coater (Polaron, model SC502) and their morphology was analyzed using a scanning electron microscope (SEM, Quanta 650, from FEI) with an accelerating voltage of 15 kV. Nanofiber average diameter and the distribution were calculated over approximately 40 fibers using SEM images (5000× magnification) and Image J software. Differential scanning calorimetry measurements (DSC) were performed in a *Perkin–Elmer* Pyris-1 apparatus at a heating rate of 10 °C.min^{-1}. Samples for the DSC studies were cut into small pieces from the middle region of the electrospun membranes and placed in 40 μL aluminum pans. All experiments were performed under a nitrogen purge. The thermal degradation kinetics of the samples was characterized by thermogravimetric analysis (TGA) in a *Perkin–Elmer* Pyris-1 TGA apparatus at heating rate scans from 10 °C.min-1 up to 40 °C.min^{-1} performed under a nitrogen atmosphere.

3. Results and discussion

The as-spun membranes (Fig. 1) show randomly oriented fibers with an average diameter of 240 ± 58 nm, with smooth surface and without bead defects (Fig. 1a). Since gelatin is a water soluble material, as-spun fiber membranes will partially or totally dissolve when in contact with an aqueous medium or high moisture environments [12]. To broaden the applications of these gelatin fibers to procedures requiring contact with water or biological medium gelatin fibers were exposed to a saturated atmosphere of GA in order to promote chemical cross-linking of the material. It was found that this chemical treatment does not influence the fiber average diameter for an exposure time up to 48 h [28]. Furthermore, the cross-linked fibers showed the same appearance as the as-spun ones (Fig. 1b). Similar behavior has been reported for other polymers such as chitosan [29].

Chemical cross-linking of gelatin with GA involves the reaction of ε-amino groups of Lys (lysine) or Arg (arginine)

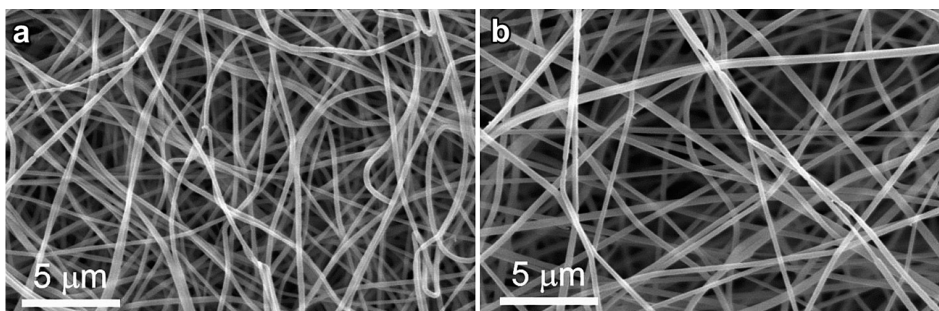

Fig. 1. Morphology of as-spun fish gelatin membranes: a) Before and b) After cross-linking with GA vapor phase during 48 h. samples were collected with an applied electric field of 1.25 kV.cm^{-1}, a feed rate of 0.2 mL.h^{-1} and a needle inner diameter of 0.50 mm.

of the polypeptide chains with aldehyde groups of the cross-linking agent by a Schiff base reaction [30]. Moreover, cross-linking is typically associated with a color change from white to pale yellow [12].

3.1. Hydrolytic degradation

Rosellini et al. [23] showed that *in vitro* degradation behavior at 37 °C of gelatin cross-linked with GA films in PBS is quite similar to that obtained in PBS collagenase solution. Electrospun cross-linked samples were immersed in PBS solution and the extent of hydrolytic degradation was determined (Fig. 2). It was found that cross-linked electrospun gelatin fibers took less than 5 min to reach the equilibrium swelling state (data not shown) and can uptake as much as 14 times of its initial mass [28]. Gelatin weight loss occurred rapidly during the first week of incubation, losing around 50% of the sample initial mass (Fig. 2a) after approximately 9 days. After 15 days, a residual mass of 32% remained. Zhou et al. [31] suggested that gelatin mass loss during *in vitro* and *in vivo* degradation occurs primarily due to solvation and depolymerization of polymeric chains. It was observed also that electrospun cross-linked membranes still showed integrity after a week *in vitro* degradation (Fig. 2b and c).

3.2. Thermal degradation

Thermal degradation of gelatin occurs in three different stages (Fig. 3). The first stage occurs at temperatures from room temperature up to 200 °C with a mass loss of around 12%. This is related to the loss of adsorbed water due to the handling of the sample at room conditions without any special care and, consequently, these data were not considered for the determination of the kinetic parameters [25,32]. Water bound to protein may be divided into three types according to its state: a) water bound by high-energy sorption centers that occurs inside collagen triple helix and which plays a major role in its stabilization by intramolecular bonds, the amount of water depending on the degree of helicity of the macromolecules. b) water absorbed by polar groups of gelatin and collagen macromolecules, bound to proteins by hydrogen bonds, located outside the helical fragments and also contributing to the stabilization of the collagen helical structure. The amount of this water in gelatin probably corresponds to the so-called monomolecular layer and can be considered as structural water. c) water absorbed by proteins to give polymolecular layers,

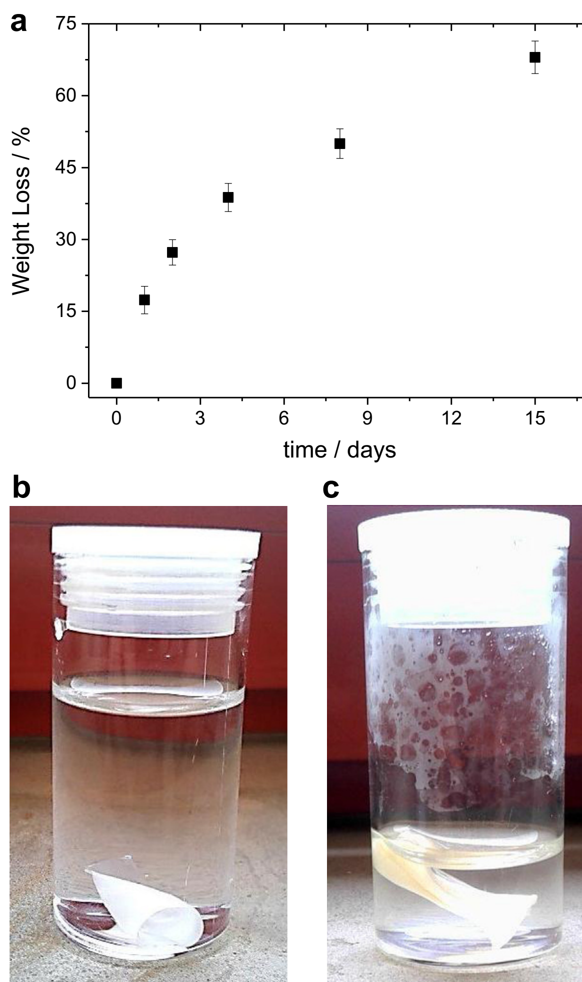

Fig. 2. a) Hydrolytic degradation kinetics for the electrospun gelatin, b) Gelatin membranes immersed in PBS for 1 h at 37 °C and c) Gelatin membranes immersed in PBS for 7 days at 37 °C.

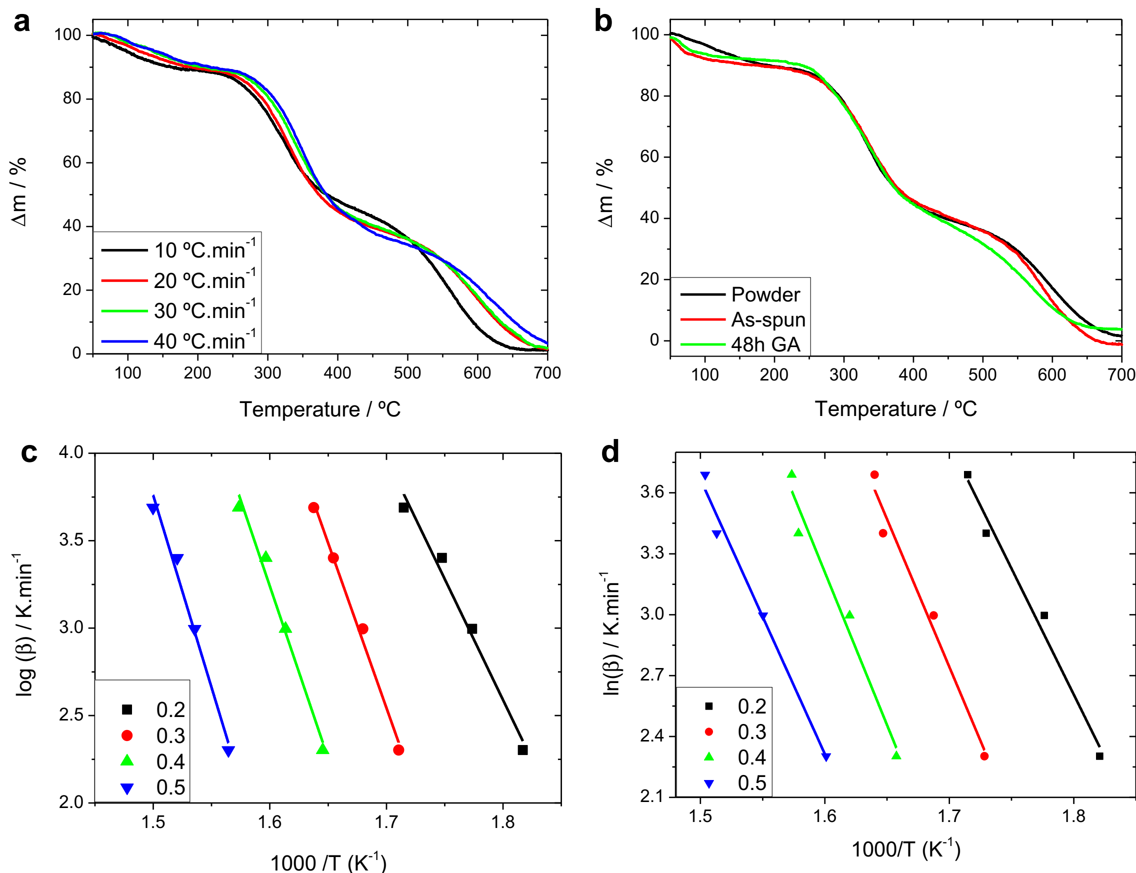

Fig. 3. TGA thermograms for a) gelatin powder, obtained at different heating rates; b) Electrospun membranes before and after different cross-linking periods, collected at a rate of 20 °C.min^{-1}; c) OFW fitting for fish gelatin powder and d) OFW fitting obtained for the gelatin fiber membranes cross-linked with GA during 48 h for different conversions.

consisting of the total amount of water bound in gelatin and the amount of structural water [33] (Fig. 4).

The second degradation stage occurs between 200 and 400 °C and is associated with protein degradation [25],

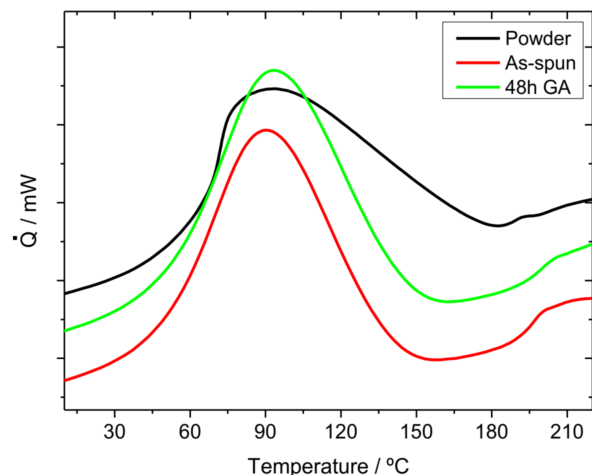

Fig. 4. DSC curves of gelatin raw material, as-spun and cross-linked with GA for 48 h.

Finally, the third stage occurs between 400 and 600 °C and corresponds to the thermal decomposition of the gelatin networks (Fig. 3) [26]. In general, the thermal stability of the gelatin electrospun membranes is similar to that of the original powder (Fig. 3b).

The kinetics of the mass loss was studied through the analysis of experiments performed at different heating rates. As expected, increasing heating rates shift the onset temperature of the degradation processes to higher temperatures, not affecting any of the main characteristics of the process itself. A model for the decomposition kinetics has the following general form:

$$\frac{\partial\alpha(t)}{\partial t} = k(T)f[\alpha(t)] \quad (2)$$

where, α represents the degree of conversion of the sample under degradation, defined by:

$$\alpha = (w_0 - w_t)/(w_0 - w_\infty) \quad (3)$$

where w_0, w_t and w_∞ are the sample weights before degradation, at a given time t and after complete degradation, respectively. The rate constant $k(t)$ changes with the absolute temperature according to the Arrhenius equation (Eq. (2)). $f(\alpha)$ represents the net result of elementary steps,

as the polymer degradation is often a chain reaction. For the reaction model, $f(\alpha)=(1-\alpha)^n$ where n is the reaction order assumed to remain constant during the degradation process.

The Ozawa-Flynn-Wall (OFW) [34,35] method assumes that the conversion function $f(\alpha)$ does not change with the heating rate for all values of the degree of conversion α. This involves measuring the temperatures corresponding to fixed values of α from the experiments performed at different heating rates, β. In this theory:

$$\ln(\beta) = \frac{\ln(AE_{act})}{R} - \ln[f(\alpha)] - \frac{E_{act}}{RT} \quad (4)$$

where A is a pre-exponential factor (min^{-1}), R is the gas constant (8.31 $J.mol^{-1}.K^{-1}$), and E_{act} is the activation energy of the degradation process. By plotting $ln(\beta)$ *vs* $1/T$, the activation energy can be obtained, regardless of the reaction order of the system (Fig. 3c and d). The validity of this model is based in the assumption that the conversion is constant for different heating rates[34,35].

The activation energy was calculated for the raw material and for the as-spun membranes before and after cross-linking with GA (Table 1). The values show that dissolution and processing conditions does not affect the thermal degradation of the material. It was previously reported that the [25] thermal activation energy for gelatin is between 175 and 275 $kJ.mol^{-1}$, the difference to the present investigation being attributed to the gelatin source, *i.e.* animal or fish gelatin, and molecular weight. After cross-linking, a decrease of E_{act} was observed in both transition stages of the electrospun membranes (Table 1), which was probably due to random scission of polymer chains [25]. GA cross-linking of gelatin promotes water insoluble enzymes, cross-linking being associated with more rigid molecules with less ordered structures. Protein–protein interaction is, therefore, apparently affected by chemical cross-linking, and decreases the thermal stability of gelatin electrospun membranes. During heating, the initially ordered structure of the samples is gradually destroyed after breaking of inter- and intra-molecular hydrogen bonds, which are responsible for the maintenance of the polymeric chain [25,27].

Thermal properties of gelatin raw materials and electrospun membranes were also analysed by differential scanning calorimetry (DSC). DSC curves obtained for the different gelatin membranes showed similar trends, indicating that electrospinning and cross-linking with GA processes do not influence gelatin thermal properties. A broad endothermic peak was observed in the range of temperatures between 30 and 180 °C, which is associated with the evaporation of bound water that was previously absorbed by the polymer membrane when in contact with air. TGA experiments showed that almost 12% of water can be absorbed by gelatin (Fig. 3) when in contact with air. Further, at 190 °C an endothermic shoulder was observed, related to the helix–coil transition, i.e., the electrospinning process does not alter the formation of the characteristic helical structure between the gelatin chains [36].

Table 1
Evolution of E_{act} for the fish gelatin powder and electrospun membranes before and after GA cross-linking.

Sample	$E_{act}/kJ.mol^{-1}$	
	First-step	Second-step
Powder	175 ± 27	177 ± 28
As-spun	175 ± 35	189 ± 35
48 h-GA	110 ± 13	110 ± 15

4. Conclusions

Fish gelatin membranes were electrospun into random fiber membranes with an average diameter of 240 ± 58 nm. In order to improve stability in aqueous environments, chemical cross-linking with GA was performed for a 48 h period, and it was found that the cross-linking treatment does not influence the fiber average diameter of gelatin membranes.

Hydrolytic degradation of gelatin membranes was performed in a PBS solution at 37 °C, and after 15 days a weight loss of 68% was observed, attributed to solvation and depolymerization of the main polymer main chains. TGA studies of gelatin powder and electrospun membranes showed similar patterns and it was found that the electrospinning processing conditions do not affect the thermal degradation of the material. Further, a decrease of the activation energy was observed for the electrospun samples as compared to the raw protein. Cross-linking leads, therefore, to a more disordered structure and to the formation of polymer networks. Finally, it was found that electrospinning process does not influence the formation of the characteristic helical structure between the gelatin chains.

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