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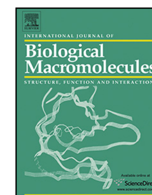

Morphological and structural investigation of wool-derived keratin nanofibres crosslinked by thermal treatment

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ABSTRACT

Mats of wool-derived keratin nanofibre have been prepared by electrospinning solutions of keratin in formic acid at 20 and 15 wt.%, and obtaining nanofibres with mean diameter of about 400 and 250 nm, respectively. These mats can find applications in tissue engineering (they can mimic the native extracellular matrix) and in wastewater treatment (they can trap small particles and adsorb heavy-metals). A drawback to overcome is their solubility in water. A stabilization method, based on a thermal treatment alternative to the use of formaldehyde, is proposed. The solubility test in the dithiothreitol/urea extraction buffer, the amino acid composition analysis and studies on keratin secondary structures suggest that the improved stability in water of thermally treated mats can be ascribed to the formation of amide bonds between acid and basic groups of some amino acid side chains.

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1. Introduction

In recent years, the electrospinning process has gained increasing attention because it is a simple and effective method to produce ultrafine fibres and nanostructured fibrous membranes.

Generally, the basic electrospinning apparatus is made of a high voltage supply, a dope flow rate controller and a metal collector. A high voltage is applied to the polymer solution droplet at the capillary tip; when the electrostatic forces overcome the surface tension of the polymer solution, a charged jet is ejected towards the collector. Before reaching the collector, the solvent evaporates and the polymer is collected in the collector as an interconnected web of nanofibres [1].

Peculiar properties of nanofibrous materials as high surface to volume ratio and high porosity, make them promising candidates for several applications such as filter membranes [1,2], wound dressing [3], cell-growth scaffolds [4], vascular grafts and drug delivery [5]. In the field of tissue engineering, the production of nanofibre from biopolymer solutions has been widely considered in order to prepare biocompatible 3D scaffolds able to mimic native extracellular matrices (ECMs) [6,7]. The biopolymers considered are carbohydrates (such as chitosan [8] and chitin [7]) or proteins (such as fibroin [9], collagen[10], and keratin [11]).

Among the biopolymers, keratin is a fibrous protein most abundant in nature being the component of wool, hairs, feathers, horns and nails [12]. At molecular level, keratin is distinguished from other proteins (as fibroin or collagen) by the high concentration of sulphur containing amino acid cysteine (7–20% number of total amino acid residues) [13]. The cysteine residues are present in the oxidized form of cystine residues, characterized by disulphide covalent bonds which confer to the protein mechanical, thermal and chemical stability. In particular, the keratins extracted from wool can be classified in two groups: intermediate filament proteins (IFPs) and matrix proteins. The IFPs are referred to as the low-sulphur keratins (LS keratins), they have molecular weights in the range of 40–60 kDa and an α-helix structure. The matrix proteins are the high-sulphur keratins (HS keratins) and they have molecular weights in the range of 11–26 kDa and mainly amorphous structure. The matrix keratins surround the IFPs keratins and interact with them through intermolecular disulphide bonds [14].

It has been demonstrated that keratins extracted from wool are biodegradable in vitro (by trypsin) and in vivo (by subcutaneous embedding in mice) [15]; moreover, they support the growth and adhesion of fibroblasts [16] and osteoblasts [17].

Despite the encouraging results on the fabrication of protein-based nanofibrous membranes, one of the drawbacks to overcome is the poor stability and structural integrity of these membranes

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Table 1
Electrospinning process conditions.

Keratin concentration [wt.%]	15	20
Electric field [kV cm^{-1}]	1.7	1.7
Tip to target distance [cm]	15	15
Flow rate [ml min^{-1}]	0.003	0.005
Time [min]	60	20
Temperature [°C]	21–25	
Relative humidity [%]	45–50	

in an aqueous environment. Several strategies have been proposed such as treatments with formaldehyde vapour [18], aqueous alcohol solution [19], water vapour [20], or crosslinking agents as genipin [6].

In this work, pure keratin nanofibre mats made of keratin extracted from wool were prepared by electrospinning the keratin dissolved in formic acid at different concentrations. The stability in water of these mats was improved by a thermal treatment and this novel stabilization process was compared with the widely used treatment with formaldehyde vapour. Moreover, the chemical and structural characteristics of untreated and thermally treated keratin nanofibres were studied and compared with those of film prepared by casting the same solutions used in the electrospinning process.

2. Materials and methods

2.1. Materials

Merino wool (21 μm fineness) in the form of tops (the sliver obtained from raw wool by scouring, carding and combing processes) was supplied by The Woolmark Company (Italy). All chemicals were reagent grade and purchased from Sigma–Aldrich, except otherwise specified.

2.2. Production of keratin nanofibres by electrospinning and keratin film by casting

Keratin protein was extracted from wool by sulphitolysis with sodium metabisulphite [21] as described in a previous work [18]. Briefly, wool fibres, cleaned by Soxhlet with petroleum ether to remove fatty matter, washed with distilled water and conditioned at 20 °C, 65% RH, were cut into snippets of some millimetres and treated with a solution containing urea (8 M) and $Na_2S_2O_5$ (0.5 M), adjusted to pH 6.5 with NaOH (5 M), using a 1:20 liquor ratio, under shaking for 2 h at 65 °C.

The mixture was filtered using, in succession, 120 μm, 30 μm and 5 μm pore size filters. The filtrate was dialyzed against distilled water using a cellulose tube (3.5 kDa molecular weight cut-off) for 3 days at room temperature, changing the distilled water four times a day. The keratin aqueous solution obtained after dialysis was freeze-dried in order to obtain pure keratin powder. Afterwards, the keratin powder was dissolved in neat formic acid at two different concentration of 15 and 20 wt.%.

For the electrospinning process, the keratin solution was placed in a 5 ml syringe with a stainless steel needle (0.2 mm internal diameter) as cathode spinneret, connected to a power supply (SL50, Spellman High Voltage Electronics Corporation, USA). The syringe was placed in a syringe pump (KDS200, KD Scientific Inc., USA) to accurately control the fluid flow fed to the spinneret. The anode (collecting screen) was a stainless steel plate. Electrospinning was performed using an apparatus in which the jet-emitting source was positioned below the grounded collector ("bottom-up") and using the operating conditions shown in Table 1. In order to increase the stability of the keratin nanofibres in aqueous environment, some keratin nanofibre mats obtained from the 20 wt.% keratin solution were treated with formaldehyde vapour overnight, and the other were heated in air at 180 °C for 2 h. Instead, keratin nanofibre mats obtained from the 15 wt.% keratin solution were subjected only to the thermal treatment (heating in air at 180 °C for 2 h). Keratin solutions at 15 wt.% were also cast onto polyethylene plates at 50 °C overnight in order to prepare keratin films.

2.3. Characterizations

Viscosity of the solutions was measured by an Anton Paar (Austria) Physica MCR 301 rheometer equipped with a PTD 200 Peltier temperature control device at 25 ± 0.1 °C with cone-plate geometry (75 mm diameter, 1° angle and 45 mm truncation). The shear rate was logarithmically increased from 1.5 to 1000 s^{-1}.

Conductivity of the solutions was measured with an Eutech Instruments (Singapore) PC 300 multi-parameter tester, calibrated with a 1413 μS cm^{-1} (at 25 °C) conductivity standard solution.

The morphology of the keratin nanofibre mats was examined by a LEO 435 VP (LEO Electron Microscopy Ltd., UK) scanning electron microscope (SEM), with acceleration voltage of 15 kV, current probe of 50 pA and working distance of about 20 mm. Samples were sputter-coated with a gold layer 20–30 nm thick in rarefied argon, using an Emitech (UK) K550 sputter coater with a current of 20 mA for 240 s. The average nanofibre diameter and its standard deviation were determined by measuring at least 100 fibres selected randomly from several mats.

The water stability tests were carried out by immersing the samples in water at room temperature for 24 h and analyzing their morphology after the immersion. The stability in water of untreated and thermally treated keratin nanofibres obtained from the 15 wt.% keratin solution was also compared with that of a film obtained by casting the same solution. In this case, the samples were immersed in water for 24 h at room temperature (20–25 °C) and the stability degree (*SD*) was evaluated by using the following equation:

$$SD \quad (\%) = \frac{W_2}{W_1} \times 100 \qquad (1)$$

where W_1 and W_2 are the weights before and after the immersion, respectively. Before weighing, the samples were dried in a oven at 105 °C for 2 h.

The solubility of untreated and thermally treated nanofibre mats in the dithiothreitol/urea extraction buffer of keratin was studied by immersing the samples in a solution of dithiothreitol containing Tris–HCl (550 mM, pH 8.6), dithiothreitol (DTT; 140 mM), ethylenediaminetetraacetic acid (EDTA; 5 mM), and urea (8 M) for 4 h under nitrogen atmosphere [22], and evaluating the concentration of the dissolved protein with the Bradford protein assay method (Bio-Rad) using bovine serum albumin as standard.

For the determination of amino acid composition, keratin powder, untreated and heated keratin nanofibres were hydrolyzed with HCl (6 N) at 110 °C for 24 h in sealed tubes. Free amino acid residues were derivatized with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC, by Waters) and eluted on a 15 cm × 0.39 cm reversed-phase column (Waters). An Alliance (Waters) high-performance liquid chromatograph (HPLC) was used, and the eluate was detected at 254 nm. The quantitative amino acid composition was determined by calibration with Amino Acid Standard H (Pierce) cysteic acid and lanthionine (TCI Europe) as external standards and α-aminobutyric acid as internal standard.

FT-IR spectra were acquired using a Thermo Nicolet Nexus Spectrometer. Samples dried at 105 °C for 1 h were mixed with KBr and pressed into pellets. 100 scans were taken in the 4000–650 cm^{-1} range and 8.0 gain. After the acquisition, the spectra were baseline-corrected and smoothed with a nine-point Savitzky–Golay function. The amide I band was resolved in Gaussian shape bands related to different protein secondary structures.

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