Materials and Design 104 (2016) 60–67

Contents lists available at ScienceDirect

Materials and Design

journal homepage: www.elsevier.com/locate/matdes

High protein content keratin/poly (ethylene oxide) nanofibers crosslinked in oxygen atmosphere and its cell culture

Jie Fan [a,d,*], Tong-Da Lei [d], Jia Li [b], Pei-Yu Zhai [d], Yong-Heng Wang [c], Fu-Yuan Cao [c], Yong Liu [a,d,*]

[a] State Key Laboratory of Separation Membranes and Membrane Processes, Tianjin Polytechnic University, Tianjin 300387, China
[b] Liaoning Provincial Key Laboratory of Functional Textile Materials, Eastern Liaoning University, Dandong 118001, China
[c] Medical Experimental Center, Jitang College, North China University of Science and Technology, 29 Zheng-tai Street, Tangshan 063300, China
[d] School of Textiles, Tianjin Polytechnic University, 399 West Binshui Road, Tianjin 300387, China

ARTICLE INFO

Article history:
Received 21 March 2016
Received in revised form 5 May 2016
Accepted 7 May 2016
Available online 10 May 2016

Keywords:
Keratin
Electrospinning
Nanofibers
Cell culture

ABSTRACT

This study focuses on preparation and structural investigation of the water insoluble Keratin/poly (ethylene oxide) (PEO) blend nanofiber mat with high content of keratin for biomedical utilization. A modified secondary crosslinking process in oxygen atmosphere was first employed to improve the water insolubility of the electrospun keratin/PEO nanofibers with high keratin content, following the primary crosslinking process with ethylene glycol diglycidyl ether (EGDE), for cell culture. A systematic quantitative analysis of liquid viscosity, FTIR, XRD, and TG were conducted to illustrate the electrospinnability of the primary crosslinked keratin/PEO blend water solution and the water insolubility of the secondary crosslinked nanofiber mat. The results indicated that the increase of the keratin molecular weight by the primary crosslinking reaction between keratin molecules by EGDE might be the main reason for the improved electrospinnability of the solution. The water insolubility of the secondary crosslinked nanofiber mat was attributed to the rebuilt of the sulfur crosslinking bonds between keratin formed in pure oxygen atmosphere during the secondary crosslinking process. The biocompatibility of the water insoluble keratin/PEO blend nanofiber mat was also investigated with cell cultivation, suggesting the nanofibers had a potential in tissue engineering and biomaterials field.

1. Introduction

Keratin, as a structural fibrous protein of hair, wool, feathers, nail, and horns of mammals, is abundantly present in wastes from the textile industry, stock farming and butchery. About 5 million tons of keratin based wastes are mostly disposed in landfills worldwide per year, since the sulfide produced during burning it for fuel may pollute the air [1]. Recently, the waste natural fibers have attracted a lot of attention for its good inhibition property against the growth of a wide variety of yeasts, fungi and bacteria, and for its excellent compatibility in biological systems. Various keratin based films and scaffolds were developed for biomedical applications [2–6].

Scaffolds with micro porous can greatly enhance their efficiency because of their large specific area. Different methods have been used to obtain porous keratin scaffold, such as phase separation and selective dissolution [2,7,8]. More recently, electrospinning nanofiber mats with nanometric pore size exhibits as the attractive materials for a wide range of conventional and high-tech applications due to its distinctly high surface area to mass ratio and high porosity [9].

However, the electrospinnability of pure keratin from water is limited because of its low molecular weight [10]. As a result, there are not sufficient chain entanglements to allow continuous fiber formation during the electrospinning process. Fortunately, this problem can be eliminated by blending with higher molecular weight biopolymers [10,11].

Poly (ethylene oxide) (PEO) is a water-soluble polymer with good biocompatibility, low toxicity and excellent spinnability. It has been proved that the fiber-forming property of keratin solution can be greatly improved by proper addition of PEO [10,12]. When PEO is blended with keratin in ratio of 30/70, the viscosity of spinning solutions increased and the spinning solution could be electrospun into continuous nanofibers with few defects. However, when keratin content is further increased, the bead-like defects would appear on the fibers. How to prepare keratin/PEO nanofiber mat with high keratin content is the urgent problem to be solved for medical applications such as tissue engineering. Xing et al. [13] proposed a method to prepare keratin/PEO nanofiber mat with high keratin content. In their method, keratin was chemically modified by reacting sulfide side group with iodoacetic acid to enhance its solubility in organic solvent. Then, the modified keratin was blended with PEO in weight ratio of 90/10 and dissolved in 2,2,2,-trifluoroethanol (TFE) for preparation of nanofibers via electrospinning. Our group also developed a novel process for making high-content keratin/PEO (90:10) blend nanofibers using two-step cross-linking process with ethylene glycol diglycidyl ether (EGDE) as cross-linker [14]. Sow et al. [15] prepared

* Corresponding authors at: State Key Laboratory of Separation Membranes and Membrane Processes, Tianjin Polytechnic University, Tianjin 300387, China.
E-mail addresses: fanjie@tjpu.edu.cn (J. Fan), liuyong@tjpu.edu.cn (Y. Liu).

http://dx.doi.org/10.1016/j.matdes.2016.05.022
0264-1275/

electrospun human keratin matrices with even larger keratin to PEO weight ratio of 60:1 from 0.5 M Na_2S aqueous solution and found it provide a bioinductive and structural environment for cell growth. More recently, Aluigi et al. [16] reported that pure keratin nanofiber had been obtained from electrospinning solutions of keratin in 98% formic acid at 20 and 15 wt.%. Although organic solvent or salt solution used in above literatures can help increase keratin content in the resulting samples or even make pure keratin nanofiber mats, however, they are either environmentally harmful or costly, which makes it a potential risk in biomedical applications.

Another problem to be solved for making biomedical keratin based nanofiber mat is that the blend or pure keratin nanofiber mats are easily to be dissolved in water because of the water solubility of keratin. Crosslinking reagents such as formaldehyde vapor, and glutaraldehyde vapor have been employed to improve the water tolerance of the nanofiber mats [13,17]. However, as reported in literatures, the nanofibers slightly lost its fibrous morphology after the crosslinking treatment, which might bring negative effect on the mechanical property of the nanofiber mat. Moreover, the chemical crosslinkers are not eco-friendly in vapor form. In order to overcome the drawbacks of cross linkers, Aluigi et al. [16,18] employed thermal treatment to improve the stability of the keratin nanofibers in aqueous environment by heating the nanofiber mat in air at 180 °C for 2 h.

In this work, the aqueous solution of keratin and PEO was primarily crosslinked to improve the electrospinnability of keratin using ethylene glycol diglycidyl ether (EGDE). Then, we suggested a modified process that the blend nanofiber mat was secondarily crosslinked in pure oxygen atmosphere to establish the three-dimensional crosslinking bridges between keratin molecules, which endowed the nanofibers with stable morphology in water. The chemical and structural characteristics of the secondary crosslinked keratin/PEO blend nanofiber mat were studied and compared with those of the uncrosslinked and the primary crosslinked samples. Moreover, the biological performance of the secondary cross-linking keratin/PEO blend nanofiber mat was investigated by cell cultivation experiment.

2. Experimental

2.1. Extraction of human hair keratin

Human hair obtained from a local hair salon was cleaned by Soxhlet with petroleum ether to remove fatty materials. The cleaned fiber was then dried at room temperature for 24 h and cut into 5 mm snippets. The extraction of keratin from human hair was performed according to the previously reported method [19]. The human hair (6 g) was shaken in 200 mL of aqueous solution containing 7 M urea, 2 wt.% SDS, and 5 wt.% $Na_2S_2O_5$ at 95 °C for 4 h. The resulting mixture was filtered through a stainless-steel mesh, and subsequently dialyzed against distilled water in cellulose tubing (molecular weight cut off = 8000–14,000 Da) for 3 days at room temperature. The dialyzed keratin solution was then concentrated with a rotary vacuum evaporator (RE-2000B, Yingyu Instrument Co., China) to 3–5 wt.%. Finally, the condensed keratin solution was lyophilized to obtain keratin powder.

2.2. Preparation of the uncrosslinked and primary crosslinked keratin/PEO spinning solution

PEO (molecular weight = 400,000 Da) power (1.5 g) was dissolved in 100 mL distilled water under continuous magnetic stirring at 45 °C for about 4 h to prepare 1.5 wt.% PEO solution, and then 0.27 g keratin powder was dissolved in 2 mL PEO solution to prepare the uncrosslinked 15 wt.% keratin/PEO (90:10) blend solution.

The primary crosslinked spinning solution was prepared by adding 0.125 mL EGDE into the above mentioned uncrosslinked keratin/PEO blend solution. The reaction mixture was stirred at 60 °C for 30 min.

2.3. Fabrication of electrospun keratin/PEO nanofiber mats

Electrospinning was performed using a horizontal set up containing a high DC voltage power supply (DW-P303-1ACFO, Dongwen high voltage supply Co., China) and a syringe pump (LSP02-1B, Longer Precision Pump Co., China). The electrospinning process was performed at 16 kV applied voltage, 15 cm working distance, and 0.5 mL/h flow rate. The nanofiber mats were produced with a deposition time of 30 min. The uncrosslinked nanofiber mat was prepared form the uncrosslinked spinning solution, while the primary crosslinked nanofiber mat was manufactured from the primary crosslinked spinning solution.

2.4. Secondary crosslinking of the primary crosslinked keratin/PEO nanofibrous mats

In order to increase the water resistance of nanofiber mat for biomedical applications, the primary crosslinked keratin/PEO nanofiber mat was further crosslinked in pure oxygen condition at room temperature for 8 days to prepare the water tolerant secondary crosslinked nanofiber mat.

2.5. Characterization

The viscosity of the spinning solutions was measured by an HAAKE RheoStress 1 rheometer at 25 °C, using 25 mm diameter plate with 1 mm gap. The shear rate was increased from 2 to 100 s^{-1}. Morphologies of the nanofiber mats were examined using a scanning electron microscope (SEM) (TM-1000, Hitachi, Japan). The chemical composition of the nanofiber mats were determined by employing a Fourier transform infrared spectroscopy (TENSOR37, Bruker, Germany) at wavelengths 400–4000 cm^{-1}. Before the spectra acquisition, the samples were dried under vacuum for 4 h. Thermal stability of the nanofiber mats were studied by using thermogravimetric analysis (TGA) (STA409PC, Netzsch, German) in the temperature range from 40 to 600 °C at the heating rate of 10 °C /min. The crystalline structure of the nanofiber mats were investigated using the diffractometer (XRD) (D8 DISCOVER, Bruker, German) with a wavelength of 0.154 nm monochromated X-ray obtained from Cu (K_α) radiation. The operating voltage and current were 40 kV and 40 mA, respectively, and the data were collected in the wide angular region from 5 to 40 $2\theta^o$.

2.6. Cell cultivation

The secondary crosslinked nanofiber mat was cut into disc-shaped pieces with 15 mm in diameter. The nanofiber mat pieces were then sterilized by immersed in 75% ethanol for 4 h and dried. After that, they were transferred to plastic cup for incubation in DMEM (dulbecco's modified eagle medium) supplemented with FBS (Fetal Bovine Serum) at 37 °C, 5% CO_2, and 95% relative humidity overnight.

Cell culture experiments were carried out using mouse fibroblasts (L929). The harvested cells (Shanghai Queen & King Biochem Co.) were resuspended in DMEM supplemented with 10% FBS, and seeded onto nanofiber mat surface and cultured in a 5% CO_2 atmosphere at 37 °C for 1 week. The cell culture medium was changed every other day.

Cell adhesion was determined by a MTT cell proliferation assay. After the seeded nanofiber mat were cultured at 37 °C under a humidified atmosphere of 5% CO_2 for 1, 2, 3 and 4 h, excess medium was removed and resupplied with 360 μL of fresh culture medium. MTT dissolved in hanks balanced salt solution (HBSS) (5 mg/mL) 40 μL was added to each well. After incubating at 37 °C for 4 h, excess medium was removed and added dimethyl sulfoxide (DMSO) to dissolve the formazan crystal. Then 100 μL DMSO solutions were injected into 96-well plates and OD value of each well was determined by auto microplate reader (Multiskan EX, Fortune lab systems, CA) while the wavelength was selected at 492 nm.