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Keratose/poly (vinyl alcohol) blended nanofibers: Fabrication and biocompatibility assessment

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ABSTRACT

Increasing interest in using keratin-based materials for biomedical application has prompted the development of keratin/PVA nanofibers. To date, several kinds of keratins (including wool, feather, and human hair reductive keratins)/PVA blended nanofibers have been fabricated but limited to the *in vitro* studies. However, few studies focused on the *in vivo* biocompatibility test of keratin/PVA nanofibers. Herein, the keratose (oxidative keratin)/PVA nanofiber, a novel type of keratin/PVA nanofiber, was fabricated with an electrospinning technique. The obtained nanofibers possess uniform fibrous structure, suitable hydrophilicity and mechanical properties, which could be affected by the mass ratio of keratose to PVA. Furthermore, the biocompatibility tests of keratose/PVA nanofibers have been performed by subcutaneous implantation into SD rats. H&E staining and Masson's trichrome staining revealed that the implants were highly compatible with body tissue, and no acute toxic effects as well as no tissue damage were observed. The implants were fully degraded within four weeks. Beyond this, the analysis of proinflammatory cytokines in rat serum indicated that the implants induced normal immune response and had no immunogenicity. These results demonstrate that keratose/PVA nanofibers have the potential for biomedical applications due to the favorable biocompatibility and biodegradability.

1. Introduction

Keratins, one of a family of fibrous structural proteins, are ubiquitous in natural polymers including human hair, wool, feather, horns, hooves and nails [1–3]. Given the unique characteristics of bioactivity, biocompatibility, biodegradability, and natural abundance [4], keratins have been fabricated into powders, hydrogels, films, sponges, dressings or some other forms for the biomedical applications [5–8], which involved hemostasis, wound healing, nerve repair, tissue regeneration and other objects [5,9–12]. Unfortunately, the poor mechanical properties of keratin-based products remain the longstanding challenge in biomedical application [13]. Therefore, researchers have undertaken to blend keratin with additional materials to enhance its mechanical properties.

Electrospinning, a prevalent method for fabricating polymeric nanofibers, has sparked interest in the biomedical field, and the prepared nanofibers commonly possess not only high specific surface area with microporous structure for cells adhesion and proliferation, but also high tensile strength for maintenance of structural integrity *in vivo*. Recently, keratin-based nanofibers have been developed in the cell culture, wound healing, bone regeneration *etc*. Some polymers, including poly (ethylene oxide), poly (vinyl alcohol), polylactic acid, polycaprolactone, and poly (lactic-co-glycolic acid) have been incorporated into the electrospun keratin nanofibers to improve its mechanical properties [14–17]. Owning to the characteristics of nontoxic, biodegradable and excellent chemical as well as physical properties, PVA has been used to blend with different kinds of keratins for the preparation of nanofibers. To date, wool keratin/PVA, feather keratin/PVA, and human hair kerateine (reductive keratin)/PVA blend fibers have been prepared by the electrospinning technique, which showed good uniformity in fiber morphology and suitable mechanical properties. Unfortunately, previous studies regarding keratin/PVA blended nanofibers had only emphasized the cellular interactions *in vitro*, such as low cytotoxicity, promoting cell proliferation, and high optical transparency [18–21]. For any novel material being developed for biomedical applications, there is a demand for further biocompatibility research *in vivo*, which have remained elusive.

Hence, we firstly prepared the human hair keratose (oxidative keratin)/PVA nanofibers by electrospinning method, and the biocompatibility of keratose/PVA nanofibers was further assessed using a subcutaneous implant model in Sprague-Dawley rats. There are some differences in the degradation, dissolve and molecular weight between the keratose and kerateine. The keratose was extracted from human hair using a method of oxidization [22], and the characteristics of keratose extracts were investigated *via* SDS-PAGE and amino acid analyses. In addition, keratose/PVA blended nanofibers were characterized by scanning electron microscope (SEM), Fourier transform infrared spectroscopy (FT-IR), contact angle and tensile strength. Finally, nanofibers were also implanted subcutaneously in Sprague-Dawley rats, and the host responses *in vivo* were evaluated by hematoxylin and eosin

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(H&E) staining, Masson's Trichrome staining and enzyme-linked immunosorbent assay (ELISA).

2. Experimental section

2.1. Materials

Human hair was obtained from local barbershop in Chongqing, China. Peracetic acid, Tris base, SDS (sodium dodecyl sulfate) and PVA were purchased from Kelong Chemical reagent Co. Ltd. (Chengdu, China). Enzyme-linked immunosorbent assay (ELISA) kits were purchased from 4A Biotech Co., Ltd. (Beijing, China). Animals were supplied by the Experimental Animal Center of the Third Military University (Chongqing, China). Unless otherwise specified, all other materials and reagents used in this study were provided by our experimental platform.

2.2. Keratose extraction from human hair

Keratose was extracted from human hair according to the oxidative method [22]. Briefly, human hair was washed with 0.5% (w/v) sodium dodecyl sulfate (SDS) to remove dust and surface grease, and dried overnight. Clean dried hair was oxidized with 2% (w/v) peracetic acid for 15 h under continuous stir, followed extracting with 0.1 M Tris base and distilled water for 2 h, respectively. Then the solution of proteins was neutralized using sodium hydroxide, centrifuged (6000 rpm for 30 min at 4 °C) to remove precipitate. The extracted solution was further concentrated and depurated using the ultrafiltration flat sheet membrane (FlowMem-0015, Filter & membrane technology, China) with 10 kDa molecular cutoff of the membrane, and human hair keratose in a powder form was achieved by lyophilization and grinding.

2.3. Characterization of human keratose

The molecular weight of keratose was determined by sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE) analysis. Electrophoretic separation of keratose was performed on 10% (w/v) polyacrylamide separating gel and 5% (w/v) polyacrylamide stacking gel system. The keratose samples were boiled for 8 min with loading buffer that containing β-mercaptoethanol to obtain denatured proteins. Then protein marker and samples were loaded into gel well, respectively. Separation was performed at 80 V for 1 h, and followed by 120 V for 2 h. Afterwards, the gels were then stained with 0.02% (w/v) Coomassie Brilliant Blue G-250 for 2 h and destained with acetic acid-ethanol solution for twice. Images were obtained by an imaging system (6000 Alfred Nobel Drive, Bio-Rad Laboratories, Inc., CA).

Quantitative amino acid analyses of keratose were performed using the method of post-column derivatization with ninhydrin by an amino acid analyzer (L-8800 Hitachi, Japan). Briefly, keratose samples ($n = 3$) were hydrolyzed with 6 N HCl in glass tubes, then hydrolyzed amino acids and ninhydrin were measured by reverse-phase HPLC.

2.4. Preparation of keratose/PVA nanofibers

Prior to preparation of keratose/PVA blended nanofibers, PVA was firstly dissolved into DI water at the concentration of 10 wt%, and keratose was dissolved into PVA solution with different mass ratios of keratose/PVA (1:1, 1:3, 1:5 and 1:7). Solution mixtures were stirred until homogeneous. Subsequently, the blend solution was supplied through a plastic syringe fitted with an 18G diameter tip that connected to a positive electrode with copper wire, and the negative electrode was attached to a metallic collector. In this study, the optimized parameters of electrospinning including voltage (9 kV), distance between tip and receptor (15 cm) and flow rate (1.0 mL/h) were fixed in the preliminary experiments. The keratose/PVA blended nanofibers were collected on nylon gauze and dried at room temperature.

2.5. Characterization of keratose/PVA nanofibers

To investigate the chemical structure of keratose and keratose/PVA blended nanofibers, Fourier transform infrared spectrum (FTIR) were performed by a FT-IR spectrometer (Thermo iN10, USA) with the wavenumber range of 400– 4000 cm^{-1}.

The surface morphologies of PVA/keratose nanofibers were observed by a scanning electron microscope (SEM) (EVOLS25, Zeiss, Germany) at an accelerating voltage of 20 kV. Prior to the SEM observation, the specimens were sputtered with gold for 35 s by using a Polaron SEM coating system. The morphologies of different mass ratios of keratin/PVA nanofibers were analyzed.

Mechanical properties of blend nanofibers with different mass ratios of keratose/PVA were measured by an extensigraph (Electro PULS E1000, Instron, USA) using a 50 N load cell with a cross-head speed of 10 mm/min at ambient conditions (room temperature, RH ~65%). All samples were prepared to cut electrospun nanofibers into a form of rectangular shape with dimensions of 40 mm × 20 mm × 2 mm (L × W × T). Samples were strained to breakage. According to the stress–strain curves, the ultimate tensile strength and elongation at break were obtained. At least five specimens for each sample were used to test mechanical properties.

The surface wettability of electrospun keratose/PVA nanofibers were measured by contact angle measurement (SDC-200,Shengding Precision Instrument Co., Ltd., China). The contact angle measurements were carried out using photology system equipped with specially microscope. A drop of water (15 μL) was dropped onto the surface of electrospun nanofiber, and adjusted the shape of water bead to measure contact angle within 20 s. The measured results were calculated and recorded by software. Nanofibers with different mass ratios of keratose/PVA were selected five samples to measure contact angle.

2.6. Animal surgical procedure

Sterile keratose/PVA electrospun nanofibers (mass ratio of 1:5) were aseptically implanted in subcutaneous tissue pockets at the dorsal of male Sprague–Dawley rats (weight of 240– 260 g). All animals were divided into experimental groups and sham groups, and all procedures were performed under the guidelines of the Institutional Animal Care and Use Committee of Chongqing University. Animals were sedated with 10% chloral hydrate by intraperitoneal injection (0.5 mL per hectogram weight), and a 10-mm full-thickness skin incision was made on each rat after unhairing with an electric razor and surface disinfection with povidone-iodine solution, and a tissue pocket was created with dissection. The implant of 15 mm × 15 mm × 2 mm (L × W × T, weight of 15 mg) was placed inside the pocket and incisions were sutured under sterile environment. Animals were euthanized at 7, 14, 21 and 28 days post-surgery ($n = 3$ rats per time point) and dissected to detach tissues for toxicity and histological examination. At the same time point, the inner side of the skin with attached implant was dissected out to assess the degradation of nanofibers. Blood samples were acquired from eyelid of rats at 1, 3, 7, 14, 21 and 28 days post-surgery to test pro-inflammatory response.

2.7. Histological examination

For histological evaluation, tissue organs (heart, liver, spleen, lung and kidney) as well as the dorsal skin of implant-site were fixed in 4% paraformaldehyde (PFA) for 48 h at 4 °C. Samples were dehydrated in increasing concentrations of ethanol, and embedded in paraffin before cutting with cross-sections (10 μm thick) on a paraffin slicing machine (CUT 4050, microTec Inc., Germany). Sections were stained with hematoxylin & eosin (H&E, Beyotime, China) and Masson's Trichrome (Beijing Leagene Biotechnology Co., Ltd., China) to assess the cellular response, vascularization and collagen deposition.