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Comparative study of kerateine and keratose based composite nanofibers for biomedical applications

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ABSTRACT

In this work, two forms of keratins, kerateine (KR) and keratose (KO), were fabricated respectively into electrospun nanofibers by combination with polyurethane (PU). The differences of the structure and material properties between KR and KO based fibers were investigated by SEM observation, ATR-FTIR, XRD, contact angle, tensile test, in vitro degradation and cytocompatibility assay. The results indicated that the KR based nanofibers exhibited a higher tensile modulus, lower fracture strain and slower degradation rate, mainly due to the reformation of disulfide crosslinking between the regenerated cysteines in KR after the reductive extraction. The KO based nanofibers demonstrated a stronger hydrophilic property and higher water uptake ability due to the cysteic acid residues resulting from the oxidative extraction. Furthermore, the combination of keratins, regardless of KR or KO, could obviously improve the cytocompatibility of PU, especially in the cell attachment stage.

1. Introduction

Keratin is a family of fibrous proteins found abundant in filamentous or hard structures, e.g., hair, wool, feathers, nails and horns [1–3]. At the molecular level, keratin features a high concentration of cysteine residues (7–20%), which form many inter- and intramolecular disulfide bonds in its natural form [3]. Besides, similar to extra cellular matrix proteins such as collagen or fibronectin, keratin contains cell adhesion motifs, arginine-glycine-aspartic acid (RGD) and leucine-aspartic acid-valine (LDV), which can support cellular attachment and proliferation [2,4–6]. These distinct structural and biological properties make keratin a focus in biomedical fields including bone regeneration [7], hemostasis [8–10], nerve regeneration [11,12], blood vessel [13], wound healing [14–16] and drug delivery [9,17–20] in the past decades.

The natural keratin is water insoluble and chemically stable due to the existing of inter- and intra-molecular disulfide bonds. Therefore, a common strategy to prepare the regenerated keratin is to break the disulfide crosslinkings and convert them into the non-crosslinked form, typically by reduction or oxidation method. The former is performed using reductive regents like 2-mercaptoethanol [21] and $Na_2S_2O_5$ [22] to cleave disulfide bonds into cysteine thiols. This producing keratin is referred as kerateine. The latter is using oxidative regents, e.g. peracetic acid [11,23] to convert disulfide bonds into cysteic acids. This oxidized keratin is called keratose. In the past decade, many forms of keratin based materials have been explored, such as hydrogel [14,15,24–26], fiber [27–30], film [22,31–34], sponge [21,35,36] and so on. We reasoned that the chemical extraction methods could influence the structure and properties of kerateine and keratose, as well as their corresponding materials. But as far as we know, few systematic researches have focused on this issue.

In this work, we made a comparative study of the structure and material properties between kerateine and keratose based biomedical material, especially in the form of nanofiber. Nanofiber features a distinctive physical configuration, i.e., three-dimensional network structure, high porosity, and high surface area-to-volume ratio, which mimics the extracellular matrix to support cell proliferation and differentiation [37–42]. To date, the electrospinning technology has been widely received as a versatile and low cost tool to construct polymeric nanofibers. Its product is usually a form of non-woven fibrous mat consisting of the fibers in the nano- to micro-scale range in the diameters [43–48]. Some efforts have been made to prepare pure keratin-based electrospun nanofibers [49]. However, the obtained fibers are too fragile to be used for practical applications [49]. To resolve this, the combination of keratin with other polymers is proved to be an advisable strategy since the composite nanofibers can obtain

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outstanding performances in both the biomedical function and mechanical properties. For example, Edwards et al. prepared a keratin/poly(Ɛ-caprolactone) (PCL) composite nanofiber with the keratin mass ratio of 0–30%. These nanofibers exhibited good uniformity, structural integrity, suitable mechanical properties, and good cellular compatibility [50]. Kim et al. fabricated a nanofibrous scaffold using polylactic acid (PLA) and hagfish thread keratin (mass ratio = 9:1) as the components. This keratin/PLA nanofiber could accelerate the viability, proliferation, and osteogenesis of MG-63 cells relative to pure PLA [51]. In recent years, polyurethanes (PU) are commonly used for biomedical applications, e.g. wound dressing [52] and blood vessel [53], due to their good flexibility. Based on this, we attempted to fabricate a keratin/PU composite electrospun nanofiber by using KR and KO as the component. The differences on their structure and physicochemical, mechanical properties as well as cytocompatibility were investigated in detail. We hope this work would supply some useful information for the wider and further applications of keratins in biomedical fields.

2. Materials and methods

2.1. Materials

Detailed information can be found in the Supporting Information.

2.2. Chemical extraction of keratose and kerateine

The raw wool, which was thoroughly washed with water and defatted with acetone by Soxhlet extraction, was adopted to prepare regenerated keratins by oxidative and reductive extraction, respectively, according to other reports with some modifications [22,54]. Briefly, for oxidative extraction, the defatted wool was treated with 2% peracetic acid at 37 °C for 10 h, while for reductive extraction, a reaction solution containing 8 mol/l urea, 0.2 mol/l SDS and 0.5 mol/l $Na_2S_2O_5$ was used at 95 °C. Detailed information can be found in Supporting Information.

2.3. Preparation of electrospun nanofibers

The keratin (KO and KR) and PU were dissolved individually in HFIP to create a 10 w/v % solution. Then, the solution mixtures with volume ratios of keratin/PU at 0/100, 25/75, 50/50 and 100/0, respectively, were blended uniformly and placed in a 10-ml syringe fitted with a stainless steel needle (0.2 mm internal diameter). The typical parameters of electrospinning were 14 kV for voltage, 20 cm for the distance between needle tip and aluminum foil receptor, and 1 ml/h for feed rate.

2.4. Characterization

Detailed information about characterization of keratins and keratin based nanofibers can be found in the Supporting Information.

2.5. Water uptake and in vitro degradation

The water uptake abilities were determined by immersing a known weight (M_o) of the nanofibers into 15 ml of physiological saline solution for 24 h at 37 °C. Then the swollen fibers were treated with filter paper until no water existed on the surface, and weighted accurately (M_w). Water uptake (%) = $(M_w - M_o) / M_o \times 100\%$. For the in vitro degradation assay, a known weight (M_o) of the nanofibers were immersed in PBS solution (pH = 7.4) for 10 days at 37 °C. Subsequently, the samples were taken out of the solution, air dried for 24 h at 40 °C, and weighted accurately (M_d). The extent of in vitro degradation (%) = $(M_o - M_d) / M_o \times 100\%$.

2.6. Cell culture

L929 mouse fibroblasts were used as the model cells and purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. They were cultured in DMEM containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C. When the cells grew up to an 80% confluency, they were trypsinized using 0.25% trypsin and resuspended at 1.0×10^4 cells/ml in fresh DMEM with FBS and antibiotics. Subsequently, 500 μl of the cell suspension was seeded onto the nanofibers, which were fitted in the 24-well plate and sterilized by UV irradiation, with the glass cover adopted as control. All seeded samples were incubated for 1–7 days at 37 °C in 5% CO_2, and half of the medium was replaced every other day.

2.7. MTT assay

After incubation for 1, 3, 5 or 7 days, the L929 cells on the samples were washed twice with PBS, and incubated in 360 μl of DMEM containing 40 μl of MTT for 4 h. Subsequently, the medium was aspirated from each well, and 400 μl of dimethyl sulfoxide (DMSO) was added. The plates were agitated gently until the formazan precipitate completely dissolved. The resulting purple solution was transferred into a 96-well plate and the optical density was determined by a microplate reader (Automated Microplate Reader, Thermo, model MK3) at 492 nm with DMSO as blank sample.

2.8. Live/dead assay

Live/Dead assay for cell viability was performed using Calcein-AM/PI double stain kit (Yeasen biotech Co., Ltd., Shanghai) according to the manufacturer's recommendation. Briefly, the cells after incubation of 5 days were treated with the Calcein-AM/PI mixture for 20 min at 37 °C, and visualized with an inverted fluorescence microscope.

2.9. Cell morphology observation

After a 3-day incubation of L929 on the samples, the cells were washed with 1 × PBS, fixed with 2.5% glutaraldehyde at 4 °C for 30 min, and washed again with 3 × PBS. Subsequently, the cells were dehydrated using an ethanol gradient (30, 50, 75, 90 and 100%) and dried in vacuum drying oven. Finally, the cells were coated with gold and observed by SEM.

2.10. Statistical analyses

All results were expressed as mean ± S.D. ($n = 3$). The significant differences were evaluated by one-way analysis of variance (ANOVA) and Student's *t*-test (Origin 7.5, OriginLab Corporation, Northampton USA). The values were considered statistically significant when $p < 0.05$.

3. Results and discussion

3.1. Molecular weight analysis and thiol content of KR and KO

Fig. 1 showed the molecular weights of the regenerated keratins, kerateine (KR) and keratose (KO) by SDS-PAGE analysis. The two proteins exhibited similar electrophoretic patterns, with three distinct bands at 10–20, 40–60 and 100–120 kDa size ranges. This result was consistent with other reports [2,55]. According to the literature, 10–20 and 40–60 kDa bands accounted for α-keratins and matrix proteins, respectively, and the 100–120 kDa band attributed to the keratin heterodimers [2,55]. Furthermore, by correlation between the color of the electrophoretic band and the protein content, it could be estimated that KR contained a larger ratio of high-molecular-weight components, e.g.