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Protocols

Feather keratin hydrogel for wound repair: Preparation, healing effect and biocompatibility evaluation

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ABSTRACT

Keratins are highly attractive for wound healing due to their inherent bioactivity, biocompatibility and physical properties. However, nearly all wound healing studies have focused on human hair keratins, and the wound-repair effects and *in vivo* biocompatibilities of feather keratins are not clear. Feather keratins are derived from chicken feathers, which are considered to be the major waste in the poultry industry, and the quality of feather keratin is easier to control than that of human hair keratin due to human hair perming and colouring-dyeing. Thus, we extracted keratins from chicken feathers, and a feather keratin hydrogel was then prepared and used to test the *in vivo* wound-healing properties and biocompatibility. The results indicated that feather keratins displayed wound-healing and biodegradation properties similar to those of human hair keratins and were also highly compatible with those of the tissue and devoid of immunogenicity and systematic toxicity. Collectively, these results suggested that feather keratin hydrogel could be used for biomedical applications, particularly effective wound healing.

1. Introduction

Wound healing is a multifaceted regenerative process that occurs in the skin and involves four stages, i.e., haemostasis, inflammation, proliferation, and remodelling [1]. Biodegradable materials have been widely used to accelerate the healing process because of their rapid haemostasis, anti-inflammation and cell proliferation-promoting properties [2–5]. An ideal biomaterial for wound healing should meet the following criteria: 1) maintain a moist environment around the wound; 2) absorb the excess wound exudate; 3) allow for gaseous exchange; 4) prevent microorganisms from infecting the wound; 5) be non-toxic, non-allergenic, and biocompatible; and 6) be easy to remove without trauma to the wound or not require removal due to biodegradability [6,7].

Keratins are cysteine-rich structural proteins derived from human hair, wool, feathers, horns, hooves and nails [8]. Due to the their unique characteristics of bioactivity, biocompatibility, biodegradability, and natural abundance, keratins have been widely developed in haemostasis and wound healing applications [9–11]. However, almost all wound healing studies have focused on human hair keratins, and the wound-repair effect and *in vivo* biocompatibility of feather keratins are not clear. Additionally, the development of feather keratin for wound healing has some advantages over human hair. On the one hand, approximately 3 billion pounds of chicken feathers are generated per year [12], but the traditional treatments for chicken feathers, i.e., recycling, including burning, burying or grinding into low quality animal feed, are not satisfactory [13]. Thus, the development of feather keratin for biomedical applications is very attractive [14]. On the other hand, the quality of human hair keratins is difficult to control because hair perming and dyeing are increasingly popular. Therefore, study of the wound healing effect and *in vivo* biocompatibility of feather keratin is necessary for the development of feather keratin-based tissue repair products.

In the present study, feather keratins were extracted and used to fabricate a hydrogel for wound healing and *in vivo* biocompatibility tests. Due to a wide range of capabilities, including a high water content to provide a moist environment for the wound, the ability to swell to absorb tissue exudates, and a porous structure that allows oxygen to permeate and supports cell attachment and proliferation, hydrogels have been widely applied in wound healing [15]. Human hair keratin, gelatine, and chitosan hydrogels have

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also been fabricated to accelerate wound repair [16–20]. Here, the characteristics of both feather keratin extracts and feather keratin hydrogels were investigated with different techniques, and the *in vivo* wound-healing effect of feather keratin hydrogel was assessed in a full-thickness excision wound healing model. Furthermore, the biocompatibility of feather keratin hydrogel was also evaluated via subcutaneous implantation in Sprague Dawley (SD) rats.

2. Experimental section

2.1. Materials

Chicken feathers were collected from the local slaughter market in Chongqing, China. Thioglycolic acid (TGA), sodium dodecyl sulphate (SDS), hydrogen peroxide (H_2O_2), hydrochloric acid and sodium hydroxide were purchased from Kelong Chemical reagent Co., Ltd. (Chengdu, China). Ellman's reagent was purchased from Enzo Life Science, Inc. (Farmingdale, New York, USA). Enzyme-linked immunosorbent assay (ELISA) kits were purchased from the Neobioscience Technology Company. The other reagents used in this study were of analytical grade.

2.2. Keratin extraction from chicken feathers

Keratins were extracted from chicken feathers according to a modified Shindai method [21]. Briefly, raw feathers were washed with 0.5% (w/v) sodium dodecyl sulphate (SDS) to remove the sand and dust. Next, the feathers were dried and cut into small filaments with lengths of 1–2 mm. The feather filaments were refluxed in EtOH solution (95%, v/v) for 2 h and pretreated with a hydrochloric acid solution (5%, v/v) for 2 h to remove the grease and break the hydrogen bonds. The clean feathers were obtained after filtering out the ethanol and HCl solution. Subsequently, the dried feathers were reduced under a nitrogen atmosphere at 50 °C for 2 h with a reaction solution that contained 0.5 mol L^{-1} TGA, 0.45 mol L^{-1} urea, 0.05 mol L^{-1} SDS, and 0.004 mol L^{-1} Tris at pH 9.0. The extractions were filtered to remove the residues, adjusted pH to 4.0, and centrifuged at 6000 rpm for 40 min at 4 °C. Finally, the obtained keratins were redissolved in sodium hydroxide (0.1 N) and dialysed using an ultrafiltration flat-sheet membrane (FlowMem-0015, Filter & Membrane Technology, China) with a 5000-Da molecular cut-off. The resulting extracts were lyophilized in a lyophilizer (FD-1A-50, Biocool Laboratory Apparatus, China) and stored.

2.3. Characterization of the feather keratin extracts

2.3.1. Thiol content assays

The thiol contents of the feather keratins were determined via Ellman's assays [22]. Briefly, 250 μL of feather keratins (1%, w/v) were added to 2.5 mL of 0.1 M PBS-EDTA reaction buffer (pH 8.0) containing 50 μL Ellman's Reagent solution (4 mg/mL). The samples were mixed and incubated at room temperature for 15 min, and the absorbance at 412 nm was then recorded with a UV–vis spectrophotometer (Alpha-1500, Shanghai Lab-Spectrum Instruments Co., Ltd, China). The thiol content was determined using a calibration curve.

2.3.2. SDS-PAGE analysis

The molecular weight of the feather keratins was determined with sodium dodecyl sulphate poly-acrylamide gel electrophoresis (SDS-PAGE) analysis. The keratin samples were dissolved in ultrapure water and boiled for 8 min with loading buffer that containing β-mercaptoethanol. The protein marker and denatured sample solution were loaded onto a precast 5–10% (w/v) gradient polyacrylamide gel system. Electrophoretic separation was performed at 80 V for 1 h followed by 120 V for 2 h. Subsequently, the gels were stained with 0.02% (w/v) Coomassie Brilliant Blue G-250 for 30 min and destained twice with an ethanol-acetic acid solution. The images were obtained with an imaging system (6000 Alfred Nobel Drive, Bio-Rad Laboratories, Inc., CA).

2.3.3. Amino acid analysis

Quantitative amino acid analyses of the feather keratins were performed using a method involving post-column derivatization with ninhydrin with an amino acid analyser (L-8800 HITACHI). The keratin samples (n = 4) were hydrolysed with 6 N HCl in glass tubes, and the hydrolysed amino acids were then measured by reverse-phase HPLC.

2.4. Preparation of the feather keratin hydrogels

Feather keratin hydrogels at various concentrations (e.g., 10, 15, and 20% (w/v)) were prepared according to the procedure summarized below. Lyophilized keratin powders and ultrapure water were added into a covered glass container at appropriate concentrations. The mixtures were mixed vigorously both by manual agitation and vortexing to achieve homogeneous distributions. Next, the desired volume of H_2O_2 solution (5 wt.% of stock solution) was added to the mixtures and mixed with mild stirring to promote the formation of disulphide bonds. The resulting mixtures were incubated overnight at 37 °C to form gels. Additionally, the feather keratin lyophilized scaffolds used in the present study were formed by freezing the 20 wt.% hydrogels at −40 °C and lyophilizing with a lyophilizer.

2.5. Characterization of the feather keratin hydrogels

2.5.1. Morphological observations

The morphologies of the lyophilized keratin hydrogels were observed with a scanning electron microscope (Nano SEM 400, FEI, USA) at an accelerating voltage of 20 kV. Prior to examination, cross-sectioned scaffold samples were placed on an adhesive stub and coated with gold under a vacuum for 30 s using a Polaron SEM coating system (681HLIBC-691PIPS, USA).

2.5.2. FT-IR analysis

The chemical structures of the feather keratins and keratin hydrogels were analysed with a FT-IR spectrometer (Thermo iN10, USA) with a wavenumber range of 400–4000 cm^{-1}. The samples used for FT-IR spectroscopic characterization were prepared by grinding the dry freeze-drying specimens with KBr at the ratio of 1:100 and pressing them to form thin disks.

2.5.3. Rheology study

The rheological analyses of the keratin hydrogels were performed on a rotational rheometer (Gemini HR nano 200, Malvern, UK) with a 20-mm plate–plate configuration. The hydrogel samples were loaded into the plate, the distance of the gap was adjusted to 0.1 mm, and the samples were scraped of excess material from the edge of the parallel plate. The oscillatory frequency sweep experiments were performed 0.1–10 Hz at a constant strain of 5% at 25 °C, and the elastic modulus (G') and viscous modulus (G") were recorded ($n = 3$).

2.5.4. Porosity test

The porosities of the keratin scaffolds were measured using a liquid displacement method with absolute ethanol [23]. In brief, the scaffold was placed in a cylinder with a certain volume (V_1) of ethanol for 30 min, and the volumes (V_2) of the liquid and liquid-impregnated scaffold were recorded. Next, the liquid-impregnated scaffold was removed, and the remaining liquid volume (V_3) was