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Development and assessment of kerateine nanoparticles for use as a hemostatic agent

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ABSTRACT

Uncontrolled bleeding frequently occurs in some emergencies which can result in severe injury and even death. Keratin hydrogel has been found that it had good ahemostatic efficacy in the previous studies. However, an ideal hemostatic agent should not require mixing or preparation in advance, and hydrogel is not easy to store and carry. In the present study, the kerateine was firstly extracted from human hair, and then was prepared nanoparticles by a modified emulsion diffusion method. The synthesized nanoparticles showed spherical morphology with an average diameter of approximately 200 nm. The results of Fourier transform infrared spectroscopy and X-ray diffraction indicated that the chemical structure of kerateine did not change but the crystal form may be transformed in the nanoparticles. In addition, kerateine nanoparticles displayed a faster clotting time in vitro study than the kerateine extracts. Furthermore, kerateine nanoparticles significantly reduced the blood loss and coagulation time in the liver puncture and tail amputation in rat models. Our results indicated that kerateine nanoparticles could quickly form a high viscosity gel onto the wound and accelerate the blood coagulation based on their high specific surface area. Therefore, kerateine nanoparticles have great potential for hemostatic application.

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1. Introduction

Uncontrolled bleeding often occurs in the emergency incidents and operating room, which results in high mortality rate [1]. It causes massive blood loss prior to receiving any medical rescue [2]. Therefore, reducing blood loss and shortening bleeding time are critical for the first aid and trauma care. An ideal hemostatic agent should have following merits; high hemostasis efficacy, easy to apply, safe, durable, wildly available, and inexpensive [3]. More and more attentions have been focused on the development of novel hemostatic agents in the recent years, and there have been many means to achieve fast hemostasis, such as powders [4], sponges [5], gels [6], dressings [7], sealants and so on [8]. These hemostatic agents contain active proteins (e.g., fibrin, thrombin and collagen) and non-protein materials (e.g., chitosan, polyethylene glycol, cellulose, bismuth subgallate, magnetic nanoparticles) [9–15], which could stimulate one or more coagulation cascades. Recently, keratins have been found to use for hemostasis in different types of injuries as a novel kind of hemostatic agents [16].

Keratins are natural proteins categorized as intermediate filaments, the cytoskeletal components of desmosome cellular junctions [17]. And keratins originated from the bulk of cytoplasmic epithelia and epidermal appendages, including hair, feather, wool, horns, hooves and nails [18, 19].Due to the excellent biocompatibility, biodegradability and cellular attachment, keratins from human hair have been used for nerve regeneration, wound healing, hemostasis, bone regeneration and cell culture [18, 20–22]. In theory, the earliest record about human hair-based keratins for hemostatic application was from a Chinese medical book called *Ming Yi Bie Lu* in the 5th century. It referred that burnt human hair could astringe leakage of blood and stop bleeding. Currently, the keratin hydrogels derived from human hair have been used as a hemostatic agent to treat the lethal liver puncture and tail amputation in animal models, which display a high improvement for blood loss [18,20]. One primary advantage of the keratin-based hemostatic agents is the ideal physical form, and the keratin hydrogels with high viscosity could adhere to the bleeding tissues [18]. However, an ideal hemostatic agent should not require mixing or preparation in advance, and hydrogel is not easy to store and carry compared to the powder forms [23].

Hemostatic powders have been widely applied. The QuikClot ®and CELOX® have been approved by the FDA and used to stop the severe bleeding. The QuikClot® would firstly absorb water after contacting with blood, and then release clotting factors and platelets in the wound, which is a key mechanism for the powders to promote blood coagulation. Keratin powder, a hydrophilic protein, would also act via the same mechanism.

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The aim of present study was to prepare the keratin nanoparticles as a novel kind of hemostatic agents. The specific surface area of keratin nanoparticles is larger than that of traditional microparticles, and then supplies a higher water uptake rate and a better film-forming property. Correspondingly, keratin nanoparticles would have a stronger hemostatic efficacy compared to keratin powders. The kerateines were extracted from human hair using a chemical reduction method firstly [24], and the characteristics of kerateine proteins were investigated via SDS-PAGE and amino acid analyses. In addition, a modified emulsion diffusion method was used to prepare the kerateine nanoparticles [25], and obtained nanoparticles were characterized by Zetasizer, scanning electron microscope (SEM), X-ray diffraction (XRD) and Fourier transform infrared spectroscopy (FT-IR). Finally, *in vitro* coagulation time and *in vivo* hemostatic efficacy of kerateine extracts (grinding into powder) and kerateine nanoparticles were also studied.

2. Materials and methods

2.1. Materials

Human hair was purchased from local barber shops in Chongqing, China. Thioglycolic acid (TGA) and glacial acetic acid were purchased from Kelong Chemical reagent Co.Ltd. (Chengdu, China). Plastic-capped glass vials were purchased from Kangjian medical apparatus Co. Ltd(Jiangsu, China). All other materials and reagents used in this study were analytical grade.

2.2. Extraction of hair proteins

Kerateines were extracted from human hair according to a modified Goddard and Michaelis method [24]: hair was firstly washed with the 0.5% SDS (*w*/*v*) to remove the surface grease and dried overnight. After then, the hair was deoxidated with 0.5 M TGA at pH 11.0 for 15 h, and the reduction process was used to break cystine bonds. The reduction solution was retained through the filtration and the crude fraction of kerateines was extracted with 100 mM Tris base solution for 2 h, followed by a second extraction using deionized (DI) water. The extractions were combined and centrifuged at 6 000 rpm for 40 min at 4 °C, and the following step was dialyzed by the ultrafiltration flat sheet membrane(FM1501, Filter & membrane technology, China) with a low molecular ultrafiltration membrane (5 000 Da). Finally, the pH and of extraction was adjusted to 7.4, and the sample was concentrated 20–30 folds on the dialysis system. The resulting extracts were lyophilized and stored.

2.3. SDS-PAGE analysis

The SDS-PAGE was performed following the previous method to separate and visualize the kerateine proteins [18]. The lyophilized kerateine powders were dissolved in ultrapure water with 5 mg/mL, and the solution mixed with 4× SDS loading buffer containing 0.6 M β-mercaptoethanol. The mixture was then heated at 100 °C for 5 min and used to prepare sample for SDS-PAGE, and the denatured solution was loaded onto precast 5%–10% gradient Tris–HCl gels. Electrophoresis was performed at 75 V for 1 h, followed by 120 V for 2 h. The gels were then stained by 0.02% (*w*/*v*) Coomassie Brilliant Blue G-250 for 2 h and distained with acetic acid twice.

2.4. Determination of amino acid profile

The amino acid analysis (AAA) of kerateines was carried out using a Waters Pico-Tag System. Briefly, kerateines were hydrolyzed using the 6 N HCl in glass tubes, produced phenylthiocarbamyl-amino acid (PTC-AA) derivatives with phenylisothiocyanate and quantified by reverse-phase HPLC.

2.5. Preparation of kerateine nanoparticles

The kerateine nanoparticles were prepared based on our previous method with some modification [25]. Kerateines were firstly dissolved into ultrapure water with different concentrations ranging from 0.25% to 1.0% (*w*/*v*), and the kerateine solutions were injected into the dilute HCl solution (pH 3.0) under sonication via a needle and supplied by a syringe pump with the speed from 0.05 to 0.25 mL/min. The ultrasonic was supplied by an ultrasonic cell disruption system (JY92-II, Scientz, China), and the power of ultrasonic cell disrupter was selected as 400 W. After the formation of kerateine nanoparticle suspension, the organic solvent was evaporated from the suspension by a rotary evaporator, and the resultant nanoparticles were lyophilized by a freeze dryer (230, Modulyod, USA) overnight and stored.

2.6. Measurement of the particle size and zeta potential

Particle size and zeta potential of kerateine nanoparticles were measured by photon correlation spectroscopy and electrophoretic laser Doppler anemometry respectively using a Zetasizer (Nano ZS90, Malvern, UK). Samples were diluted to appropriate concentrations with deionized water.

2.7. Observation of the morphology

The surface morphology of the kerateine nanoparticles was observed by SEM. A piece of nanoparticle-loaded aluminum foil was coated with gold metal under vacuum and then examined by a scanning electron microscope (EVOLS25, Zeiss, Germany).

2.8. Fourier transforms infrared spectroscopy analysis

The chemical structure of kerateine protein and kerateine nanoparticles were analyzed by a FT-IR (5DX/550II, Nicolet, USA), the samples used for the FT-IR spectroscopic characterization were prepared by grinding the dry specimens with KBr and pressing them to form disks.

2.9. X-ray diffraction analysis

The XRD experiments were carried out using an X-ray diffractometer (6000X, Shimadzu, Japan). Kerateines and kerateine nanoparticles were analyzed in the 2θ ranging from 5° to 45° with a step width of 0.04° and a count time of 2 s.

2.10. Coagulation time in vitro

The whole blood coagulation time of kerateine nanoparticles was measured according to the previous study [26]. 50 mg of lyophilized kerateine nanoparticles were portioned into glass tubes, and 1 mL of fresh rat blood was put into the tube in a water bath maintained at 37 °C. The tubes were gently tilted every 15 s until the blood/nanoparticle aggregate completely ceased to flow, and recorded the time. The whole blood clotting time of kerateine extracts was also determined as mentioned above, and the lyophilized extracts were grinded by a mortar. Meanwhile, the fresh rat blood was also put into blank tube without any additive, and the blood coagulation time was recorded as control.

2.11. Rat liver puncture and tail amputation

All procedures were performed under a protocol approved by the Chongqing University Animal Care and Use Committee (Chongqing, China). Surgeries were completed on 1 ~ 2-month-old Sprague-Dawley rats, and anesthesia was induced with 10% chloral hydrate by intraperitoneal injection (0.5 mL/100 g). Tail amputation was completed at the length of 5 cm from the tail bottom using surgical

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