In Vivo Biocompatibility and Improved Compression Strength of Reinforced Keratin/Hydroxyapatite Scaffold

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Abstract A rapid freezing/lyophilizing/reinforcing process is suggested to fabricate reinforced keratin/hydroxyapatite (HA) scaffold with improved mechanical property and biocompatibility for tissue engineering. The keratin, extracted from human hair, and HA mixture were rapidly frozen with liquid nitrogen and then lyophilized to prepare keratin/HA laminar scaffold. The scaffold was then immersed in PBS for reinforcement treatment, and followed by a second lyophilization to prepare the reinforced keratin/HA scaffold. The morphology, mechanical, chemical, crystal and thermal property of the keratin/HA scaffold were investigated by SEM, FTIR, XRD, DSC, respectively. The results showed that the keratin/HA scaffold had a high porosity of $76.17 \pm 3\%$. The maximum compressive strength and compressive modulus of the reinforced scaffold is 0.778 and 3.3 MPa respectively. Subcutaneous implantation studies in mice showed that *in vivo* the scaffold was biocompatible since the foreign body reaction seen around the implanted scaffold samples was moderate and became minimal upon increasing implantation time. These results demonstrate that the keratin/HA reinforced scaffold prepared here is promising for biomedical utilization.

Keywords Keratin · Hydroxyapatite · Scaffold · Biomaterial · Hair

1 Introduction

Keratin is a kind of the autogenous proteins, which has intrinsic biological activity, excellent degradability and good biocompatibility. Compared with other autogenous materials, keratin shows attraction in field of regenerative medicine material and comes from a variety of sources, such as hairs, feathers, nails and horns [1–4]. Among these sources, human hair that comprises about 80% protein of the total mass, which mainly consists of α -keratins with high molecular weight components [5, 6]. The human hair keratin emerges significant advantages since it has low immune rejection and no risk of transmission of diseases [7]. It has been indicated that keratin porous scaffolds support preosteoblast cells attachment, propagation and growth [2, 8, 9]. However, the low hardness and easy deformation of keratin under stress make it difficult to meet mechanical demands as biomedical material.

Hydroxyapatite (HA) is an important inorganic biomaterial for its biocompatibility and bioactivity. Moreover, it

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has unique characteristic of high surface area to volume ratio and ultra fine structure similar to biological apatite, which has a great influence on cell–biomaterial interaction [8, 10–12]. However, sintered hydroxyapatite could not serve alone as biomaterial due to its weakness in strength.

Mixing the keratin and hydroxyapatite to make reinforced hybrid biomaterials provides an effective approach to obtain scaffold with high porosity, improved mechanical property, and good biocompatibility.

Previous study [8] fabricated the HA/keratin composite sponges by immersing the keratin sponge in buffer containing both Ca and phosphate ions for few days. The obtained composite sponge had a great advantage that could act as the initial deposition and nucleation sites of the amorphous calcium phosphate from the body fluid. However, the scaffold obtained by this method had some defects such as low mechanical strength and poorly interconnected pores. George et al. [13] proposed two processes to prepare HA/keratin composite sponges with different microstructures. One is by molding to obtain dense microstructure sponge; the other method is to mix liquid nitrogen ice crystals with keratin and hydroxyapatite. Both HA/keratin composite sponges showed a good biocompatibility in animal experiments. Though these high-density sponges with high strength had a good application in bone tissue engineering, the complex fabrication process and high density might restrict their application in some biomedical fields.

In this work, we proposed a rapid process to fabricate a reinforced keratin/HA composite scaffold with required microcellular structure and mechanical properties. The chemical, structural and thermal characteristics of the reinforced scaffold were investigated. Furthermore, the biological performance of the reinforced keratin/HA composite scaffold was evaluated *in vitro* implantation in mice.

2 Materials and methods

2.1 Materials

Human hairs were collected from a nearby local hair salon. Urea, sodium dodecyl sulfate (SDS), 2-mercaptoethanol, petroleum ether, sodium hydroxide and other chemicals were commercially available with analytical grade. Hydroxyapatite (molecular weight 502.31 kDa, purity 96%) was obtained from Hualan Ltd. (ShangHai, China). The deionized water (1.8 $\mu\text{S}/\text{cm}$) was made by water purification system.

2.2 Preparation of keratin solution

The keratin was extracted from human hair according to the method reported previously [14]. Briefly, the hair fibers were

cut into 5–10 mm length, washed by petroleum ether and deionized water, and dried at room temperature. Then the cleaned hair (8 g) was mixed with extraction medium in a round-bottom flask and shaken at 60 °C for 12 h. The extraction medium was prepared by dissolving SDS (3 g), urea (37.5 g), and 2-mercaptoethanol (7 g) in 120 ml aqueous solution, which was adjusted to pH = 9 with 1 M NaOH. The resulting mixture was filtered through a 100 mesh, and the filtrate was thoroughly dialyzed against 3 l degassed water containing 2-mercaptoethanol (3.5 g) with cellulose tubing (molecular weight cutoff of 12,000–14,000 Da) for 3 days, with a change of outer solution twice a day. Then the dialysed solution was centrifuged at 5000 rpm for 15 min, and the supernatant solution was concentrated to 110 mg/ml keratin solution by rotary vacuum evaporator (RE-2000B, Gongyi Yingyu instrument factory, China). A part of the concentrated keratin solution was lyophilized as keratin powder for test.

2.3 Fabrication of keratin/HA laminar scaffold

Hydroxyapatite powder (0.6 g) was added directly into a 12 ml keratin solution with a concentration of 110 mg/ml at room temperature. The solution was thoroughly mixed by ultrasonic cell disruptor for 15 min at 60 W (XINY-IID, Ningbo XinYi Ultrasonic equipment Co., Ltd, China). After that, 5 ml solution was put into a 10 ml polypropylene tube (internal diameter 12 mm, height 35 mm) and rapidly frozen in liquid nitrogen to avoid hydroxyapatite sedimentation. Then the tube was immediately kept at $-60\text{ }^{\circ}\text{C}$ for 24 h and subjected to lyophilization for 24 h to form a cylindrical keratin/HA laminar scaffold.

2.4 Fabrication of reinforced keratin/HA scaffold

The keratin/HA laminar scaffolds were placed in sealed tubes containing 10 ml PBS (pH 7.4) and gently shaken at 37 °C in table concentrator (SHZ-82, Changzhou Kai Hang Instrument Co., Ltd. China). At different soaking intervals, e.g. 1, 3, 5 and 7 days, the scaffolds were removed from the tube and washed in 100 ml distilled water at room temperature to remove excess PBS. Then, the scaffolds were placed in 12-well culture plate, frozen at $-20\text{ }^{\circ}\text{C}$ for 5 h and lyophilized for 48 h to obtain the reinforced keratin/HA scaffold using a freeze dryer (LGJ-12, Beijing Sonyuan Huaxing technology Development Co., Ltd, China).

3 Characterizations

3.1 Gel electrophoresis

SDS–polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 10–20% gradient gel (Phase Gel Bull R,

Shanghai Klarmar Reagent Co., Ltd). Aqueous keratin solution (3 μ l) was subjected to SDS-PAGE using molecular weight marker [broad spectrum protein marker (molecular weight 11–245 Kda, PR1920, Shanghai Solarbio Bioscience and Technology Co., Ltd), and protein standard (molecular weight 14.4–116 Kda, Shanghai Beyotime Bioscience and Technology Co., Ltd)] at 200 V. The protein bands in the developed gel were stained by Coomassie brilliant blue G-250 (Shanghai Beyotime Bioscience and Technology Co., Ltd) and analyzed by means of a densitometer.

3.2 Amino acid analysis

Human hair and keratin extracted from human hair were hydrolyzed with HCl (6 N) at 110 °C for 24 h under nitrogen atmosphere. The hydrolysate was filtered into a volumetric flask and diluted to constant volume. 2 ml sample from volumetric flask was deacidified. Then, the deacidified sample were solved in 1 ml buffer with a shaker and filtered to prepare the sample for analysis. The quantitative amino acid composition, expressed as mol% for each amino acid, was determined by amino acid analyzer (A300, MembraPure, Germany).

3.3 Swelling and water uptake

A cylindrical sample (Φ 12 * 10 mm) of the reinforced keratin/HA scaffold was placed at ambient environment until the sponge reached to equilibration. Subsequently, the diameter and weight of the scaffolds were recorded. Then, the scaffolds were immersed in deionized water at different soaking intervals at 37 °C, including 1, 2, 4, 8 and 24 h. After that, each of the scaffolds was carefully removed and gently blotted with a filter paper to remove the excess water, and immediately measured the weight and diameter. Swelling ratio of the scaffold was calculated

$$S = (D_{\text{wet}} - D_{\text{dry}}) / D_{\text{dry}} \times 100\% \quad (1)$$

where D_{wet} and D_{dry} are the diameters of the swollen and dry scaffold, respectively.

Water uptake of the scaffolds was determined according to the following equation

$$W = (W_{\text{wet}} - W_{\text{dry}}) / W_{\text{dry}} \times 100\% \quad (2)$$

where W_{wet} and W_{dry} represent the weight of wet and dry scaffold, respectively.

3.4 Density and porosity

Liquid displacement method was employed to measure the porosity and density of the reinforced keratin/HA scaffold. In brief, a scaffold with a known weight W_0 was immersed

in a container with a known volume V_0 of ethanol. Then the container was placed in vacuum condition to make sure each pore of the reinforced keratin/HA scaffold was filled with ethanol. The volume of the ethanol with the scaffold was recorded as V_1 . Then, the scaffold was removed from the container and the residual ethanol volume was measured as V_2 . The density and porosity of reinforced keratin/HA scaffold was calculated according to the following equations.

$$\rho = W_0 / (V_1 - V_2) \quad (3)$$

$$\varepsilon = (V_0 - V_2) / (V_1 - V_2) \quad (4)$$

where ρ and ε are the density and porosity of reinforced scaffold, respectively.

3.5 Mechanical properties

In this study, compression modulus of the scaffolds was measured using a universal testing machine (INSTRON3369, INSTRON, America). The reinforced keratin/HA scaffold was cut into cylindrical samples of 16 mm in diameter and 3 mm in thickness. Compression modulus measurements were conducted with a crosshead speed of 0.5 mm/min at 25 °C under 65% relative humidity (RH).

3.6 Scanning electron microscopy (SEM)

Morphologies of the keratin/HA laminar scaffold and reinforced keratin/HA scaffold were observed using a scanning electron microscope (TM-1000 Hitachi, Japan) at an acceleration voltage of 15 kV.

3.7 FTIR

The chemical compositions of the HA powder, keratin powder and scaffolds were measured using a Fourier transform infrared spectroscopy (FTIR, TENSOR37, Bruker, Germany) at wavelengths 400–4000 cm^{-1} . Before the spectra acquisition, samples were dried under vacuum for 4 h.

3.8 XRD

The crystalline structures of the HA powder, keratin powder and scaffolds were investigated using a X-ray powder diffractometer (XRD) (D8 DISCOVER with GADDS, BRUKER AXS, America) 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 $2\theta = 5^\circ$ to 40° .

3.9 DSC

Thermal behaviors of the HA powder, keratin powder and scaffolds were evaluated using a Differential scanning calorimeter (DSC) (DSC7, PERKIN ELMER, America) in the temperature range from 20 to 350 °C, at 10 °C min⁻¹.

3.10 *In vivo* degradation

The animal experiments were performed under a protocol approved by the laboratory animal center of North china university of science and technology (XYXK (冀) 2015-0038). Adult male CD1 Webster mice (Medical Experimental Center, North china university of science and technology) about 35 g in weight were clipped under anaesthesia with isoflurane. After disinfection of the skin with iodine, an incision was made on the mid-portion of the back. The sterilized reinforced keratin/HA scaffolds (8 mm × 2 mm) were implanted into the subcutaneous tissue. The incisions were closed with absorbable Vicryl sutures. At selecting implantation intervals (1, 2, 3 weeks, 1 and 2 months), the mice were sacrificed by euthanasia. The harvested scaffolds were fixed in 10% formaldehyde, embedded in paraffin wax, sectioned and stained with hematoxylin and eosin (H&E) staining for histological observations.

4 Results and discussion

4.1 Identification of extracted keratins

4.1.1 SDS-PAGE analysis

The SDS-PAGE of the keratin solution showed three major bands at 27, 42, and 63 KDa (Fig. 1). The high-molecular weight fraction is derived from the microfibril and the low-molecular weight fraction from interfilament matrix [14].

4.1.2 Amino acid analysis

Table 1 shows the amino acid composition of keratin powder with respect to the amino acid composition of the original human hair. A total of 17 amino acids were identified. Tryptophan is irreversibly destroyed in the hydrolysis step and cannot be quantified. Asparagine and glutamine are completely converted to aspartic and glutamic acid, respectively. It can be seen from Table 1 that cysteine were the most abundant amino acid present in human hair and keratin extracted from human hair [15]. Cysteine (Cys), which possess a reactive mercapto group (–SH), is a kind of characteristic amino acid in keratin. The –SH groups can easily form disulfide crosslink bonds

Fig. 1 SDS-PAGE: **A** protein standard; **B** broad spectrum protein marker; **C** keratin extracted from human hair

Table 1 Amino acid analysis of human hair and extracted keratin from human hair

Amino acid	Human hair (Mol%)	Keratin powder (Mol%)
Asp	6.004	5.619
Thr	4.834	3.695
Ser	7.928	5.821
Glu	12.368	10.229
Gly	2.914	1.906
Ala	2.855	1.841
Cys	26.861	14.573
Val	4.215	3.416
Met	0.399	0.588
Ile	2.468	1.91
Leu	5.237	4.935
Tyr	1.657	2.655
Phe	2.124	1.47
His	1.103	0.914
Lys	2.8	1.058
Arg	7.754	6.496
Pro	4.279	3.161

Values are expressed as the mol% of the total identifiable amino acids

between keratin molecules by oxidation, which significantly affect the property of keratin material. The cysteine component (Cys)2 in the original human hair (26.86%) is about two times higher than that in the keratin (14.57%) derived from human hair. We deduced that the significant

decrease of the cysteine component in the keratin powder is due to the oxidation of $-SH$ group of the cysteine by air, since $-SH$ group on the cysteine obtained by reduction are reactive and can be easily oxidized by the oxygen in air forming cysteic acid. The experimental result suggested that a large amount of cysteine obtained by reduction reaction was oxidized in air (see FTIR result). The residual cysteine may be coupled forming new crosslinking bonds between keratin moleculars in the reinforced keratin/HA scaffold. Thus, the reinforced keratin/HA scaffold gained the non-water soluble property.

4.2 Morphologies of keratin/HA scaffolds

Figure 2 shows the SEM micrographs of the keratin/HA laminar scaffold and the reinforced keratin/HA scaffold. The HA particles are evenly dispersed in both of the

Fig. 2 Scanning electron micrographs of **A** keratin/HA laminar scaffold **B** reinforced keratin/HA scaffold

scaffolds. The keratin/HA laminar scaffold (Fig. 2A) has a laminar structure due to the fast freezing process in liquid nitrogen. Moreover, the fast freezing rate also affects the pore size of the lyophilized scaffold [16, 17]. During the freezing process in liquid nitrogen, there is a large temperature gradient along the direction of heat flow. Thus, the ice crystal formed during the frozen process is parallel to the direction of the heat flow [18]. Consequently, the keratin/HA laminar scaffold has a lamellar pore structure.

The reinforced keratin/HA scaffold exhibits the open pore structure (Fig. 2B) with good porous interconnectivity. The formation of the open pore structure is attributed to the high swelling effect of the keratin/HA laminar scaffold by immersing the scaffold in PBS. When the keratin/HA laminar scaffold was immersed in PBS, the scaffold absorbed water and began to swell. Then the laminar pore opened. The open pore structure was then fixed and retained in reinforced keratin/HA scaffold by the following freeze drying process.

4.3 Swelling and water uptake

The swelling-time curve of the reinforced keratin/HA porous sponge is shown in Fig. 3. A rapid swelling occurred during the first few hours after the scaffold was immersed in water. The swelling ratio of the scaffold reaches about 24% in 5 h. Then, the swelling rate gradually decreased after 8 h with a near saturated swelling ratio of 25% at 25 h.

The water uptake percentage of the sponge after immersing in water for 24 h was calculated to be $822 \pm 15\%$. The high value of water uptake percentage is due to the large swelling ratio and porosity in the scaffold.

Fig. 3 Swelling-time curve of the reinforced keratin/HA scaffold

4.4 Density and porosity

The density of the reinforced keratin/HA scaffold is calculated to be 0.0712 g/cm^3 , and the porosity is $76.17 \pm 3\%$. The high porosity of the scaffold provides a necessary condition for cell growth.

4.5 Mechanical properties

Even though it has been reported that the mechanical property of keratin based material can be improved by mixing hydroxyapatite powder with keratin in different ways [13], the lyophilized keratin/HA laminar scaffold was still too fragile to measure its compressive mechanical properties. To improve the mechanical properties of the keratin/HA laminar scaffold, a reinforcement was conducted by immersing the scaffold in the PBS (pH = 7.4) and followed with lyophilization. The compression strength and Young's modulus of the reinforced keratin/HA scaffold with different immersion times were shown in

Fig. 4 **A** Compression strength and **B** Young's modulus of the reinforced keratin/HA scaffold with different time of immersion in PBS

Fig. 3. It can be seen that the reinforced keratin/HA scaffold has the largest compressive strength of 0.778 MPa (Fig. 4A) and Young's modulus of 3.3 MPa (Fig. 4B) after soaking in PBS for 1 day. With longer immersion, the compression strength and modulus of the obtained scaffolds decrease. That is because the prolonged immersion can lead to the degradation of keratin [19], which has a negative impact on the mechanical properties of the scaffold.

4.6 FTIR

Figure 5 shows the FTIR spectra of keratin powder, HA powder, keratin/HA laminar scaffold and reinforced keratin/HA scaffold. Figure 5a exhibits all the characteristic peaks of keratin, the stretching vibrational absorption band at 3276 and 2913 cm^{-1} are assigned to the N–H bond and C–N bond, respectively. The strongest absorption peak at 1641 cm^{-1} is assigned to the C=O stretching vibration of amide I band, which is especially sensitive to the secondary structure of the protein [20]. Another major peak at 1527 cm^{-1} represents N–H bending vibrational bands of amide II. The broad peak related to N–H and C–N stretching vibration of amide III is presented in the region of $1221\text{--}1236 \text{ cm}^{-1}$, and the peak at 1043 cm^{-1} denotes S–O stretching vibration FTIR [20, 21]. The FTIR spectrum of HA powder is shown in Fig. 5b, the characteristic peaks corresponding to the phosphate functional groups (PO_4) are detected at 1092 , 1023 and 963 cm^{-1} , which are all assigned to the P–O stretching vibrational bands [22].

Figure 5c, d are the FTIR absorption spectra of keratin/HA laminar scaffold and the reinforced keratin/HA scaffold, respectively. These two spectra have a quite similar

Fig. 5 FTIR spectrum of samples (a) keratin powder; (b) HA powder; (c) keratin/HA laminar scaffold; (d) reinforced keratin/HA scaffold

Fig. 6 X-ray diffraction curves of the samples (a) keratin powder; (b) HA powder; (c) keratin/HA laminar scaffold; (d) reinforced keratin/HA scaffold

Table 2 Crystallinities of keratin, HA/keratin laminar scaffold and reinforced HA/keratin scaffold

Sample	Crystallinity (%)
Keratin powder	20.54
HA/keratin laminar scaffold	45.43
reinforced HA/keratin scaffold	61.90

appearance with respect to functional chemical groups. Compared with the spectra of pure keratin spectrum, the peak of hydroxyl group vibrational band at 3250–3500 cm^{-1} in the spectra of the keratin/HA laminar scaffold and reinforced keratin/HA scaffold are broader and stronger, which is due to the crystal water in the HA powder [23]. The amide I bands at 1500–1600 cm^{-1} in the spectrum of the reinforced keratin/HA scaffold is weaker than that of the keratin/HA laminar scaffold, suggesting that keratin decomposition occurred in PBS during the reinforcing treatment [6]. In contrast, the peak strength of the phosphate groups at 1000–1100 cm^{-1} are intensified in the spectrum of the reinforced keratin/HA scaffold, which indicates the phosphate group content in the reinforced HA/keratin scaffold increased after immersing in PBS (pH = 7.4). The increase of phosphate group is due to the growth of calcium phosphate crystal in PBS [23]. It has been reported that when HA composite materials are immersed in PBS, the HA will dissolve. Accordingly, the Ca^{2+} concentration increases [22]. The calcium ions are considered to have electro-static interaction with the α -keratin molecules [6]. The carboxyls of keratin trap calcium ions to form chelate of calcium ion, which support the

Fig. 7 DSC thermograms of samples (a) pure keratin; (b) keratin/HA laminar scaffold; (c) reinforced keratin/HA scaffold; (d) HA powder

nucleation site for growth of calcium phosphate crystal in PBS [8]. Since inorganic reaction between Ca^{2+} and HPO_4^{2-} usually yields multiple compositions of calcium phosphate [6], it can be deduced that the newly formed calcium phosphate crystal may have different crystalline forms with higher ratio of PO_4^{2-} to Ca^{2+} . That would be the reason for the increase of phosphate group. It is the formation of calcium phosphate crystal in PBS and further growth of calcium phosphate crystal that result in the improved mechanical property of the reinforced keratin/HA scaffold. In addition, the open pore structure of the reinforced keratin/HA scaffold is also positive to improve the mechanical property of the reinforced scaffold comparing with the laminar pore structure.

4.7 XRD

The crystalline structures of the samples were investigated by X-ray diffraction and shown in Fig. 6. The X-ray diffraction peaks of keratin powder located at $2\theta = 10^\circ$ and 20° in Fig. 6a correspond to α -helix structure and β -folded structure, respectively. Figure 6b shows the typical sharp diffraction peaks of HA powder at $2\theta = 25.8^\circ$ (002) and 31.9° (211) [24]. Figure 6c shows both the characteristic diffraction peaks of keratin and HA, indicating that the addition of keratin does not change crystallographic structure of HA in the keratin/HA composite scaffold. The characteristic diffraction peaks of β -sheet structure in the spectrum of the keratin/HA laminar scaffold gradually weakens after being immersed in PBS, as shown in Fig. 6c, d, which suggests that part of keratin might have been degraded during the reinforcement treatment in PBS. The diffraction peaks at $2\theta = 31.9^\circ$ is intensified in the

Fig. 8 Histological section of the reinforced keratin/HA scaffold after implanted for **A** 1 week (Cells of a few cell layers thick were accumulated on the periphery of the scaffold, see solid black arrow. Cells migrated toward the inner part of the scaffold, see black arrow.)

B 1 week **C** 2 weeks, **D** 3 weeks, **E** 1 month (cells migrated into inner portion scaffold, see black arrow.) and **F** 2 months (cells migrated into inner portion scaffold, see black arrow.)

diffraction spectrum of the reinforced keratin/HA scaffold, indicating that the amount of calcium phosphate increased after reinforcing treatment in PBS.

Table 2 listed the crystallinities of the samples calculated from the X-ray diffraction curves using JADE software. The results show that the crystallinity increases from 20.54% for the keratin powder to 45.43% for the HA/

keratin laminar scaffold due to introduction of the crystal HA powder in the composite scaffold. After immersing in PBS, the crystallinity of the reinforced HA/keratin scaffold further increased to 61.90%. Crystallinity result of the scaffold indicated that the growth of calcium phosphate occurred during the reinforcing treatment process, and the result is consistent with the FTIR analysis.

4.8 Thermal behavior

Figure 7 shows the DSC thermograms recorded in the temperature range from 25 to 350 °C. The obvious endothermic peaks below 100 °C in Fig. 7a–c are due to water evaporation. In the thermogram of keratin powder (Fig. 7a), the endotherm peak at 235 °C is related to the denaturation of the keratin [25]. In the thermogram of keratin/HA laminar scaffold sample (Fig. 7b), the protein denaturation peak shifts to lower temperatures at 205 °C. This behavior suggests that the interaction between HA powder and keratin self-assembling may cause the decrease of the thermogram stability of keratin [8]. After immersing in PBS, the protein denaturation peak of the reinforced keratin/HA scaffold sample rises to a higher temperature at 232 °C (Fig. 7c). The improved thermal denaturation temperature of keratin indicates that the growth of calcium phosphate crystal induced by the chelate of calcium ion fixed to the keratin endows keratin molecules with a thermally more stable secondary structure.

The endotherm peaks corresponding to keratin degradation are located at 322, 276, and 320 °C in the thermograms of pure keratin, keratin/HA laminar scaffold, and reinforced keratin/HA scaffold (Fig. 7a–c), respectively. The degradation temperature variation of keratin for the three samples is in consistent with that of the denaturation temperature of keratin. There are no obvious endotherm peaks in the thermogram of HA powder (Fig. 7d), indicating that the melting temperature of HA powder is higher than 350 °C.

4.9 *In vivo* biocompatibility

Figure 8 is the histological section of the reinforced keratin/HA scaffold after implantation in mice for 1, 2, 3 weeks, 1 and 2 months. There are some cells accumulated on the periphery of the scaffold (see Fig. 8A, solid black arrow). Several cells migrated toward the inner part of the scaffold (see Fig. 8A, black arrow), but did not reach the inner part of the scaffold (see Fig. 8B). There is no cell observed in the inner portion of the scaffold after 2 and 3 weeks implantation (Fig. 8C, D). Only very few cells are observed immigrated into the inner portion of the scaffold after 1 and 2 months postoperatively (Fig. 8E, F, black arrow). The results demonstrated that the foreign body reaction only occurred around the implanted scaffold samples.

The porous structure of the scaffold almost remained the same in the first 3 weeks postoperatively. After 3 weeks of implantation, the degradation of the entire pore architecture became obvious. The blank area gradually enlarged as the keratin material was absorbed and replaced by tissue

(Fig. 8D–F). The degradation process of reinforced keratin/HA scaffold did not show gross inflammation.

In conclusion, a novel reinforced keratin/HA scaffold with high mechanical property and high porosity was prepared by the rapid freezing/lyophilizing/reinforcing method. The scaffold is made from keratin extracted from human hair with molecular weight of 27, 42, and 63 KDa. Human hair has a high cysteine component of 26.86%. About half of the cysteine component in the original human hair were oxidized in the form of cysteic acid during the extraction process.

The structural characterization showed that the reinforced keratin/HA scaffold has an open pore structure with porosity of $76.17 \pm 3\%$, and density of 0.0712 g/cm^3 . The swelling ratio of the scaffold reached about 24% in 5 h and the water uptake percentage of the sponge was calculated to be $822 \pm 15\%$ after immersing in water for 24 h.

Mechanical property test showed that the reinforced keratin/HA scaffold has a compressive strength of 0.778 MPa and Young's modulus of 3.3 MPa after soaking in PBS for 1 day.

FT-IR analysis suggested that the strong hydrogen bonding interaction and chelate of calcium ion formed between keratin and HA is beneficial to improve the mechanical property of the keratin/HA laminar scaffold. Furthermore, the growth of calcium phosphate crystal by immersing in PBS can further improve the mechanical property of the reinforced keratin/HA scaffold, even though the keratin partially decomposed during the PBS reinforcement treatment. This finding was confirmed by XRD and DSC analysis.

Subcutaneous implantation studies in mice suggested that the reinforced keratin/HA scaffold has an excellent biocompatibility and biodegradation performance. The improved mechanical property and biological performance of reinforced keratin/HA scaffold shows that it is a promising biomedical material.

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Compliance with ethical standards

Conflict of interest We wish to confirm that there are no known conflicts of interest associated with this publication.

Ethical statement This study was approved by the laboratory animal center of North china university of science and technology (XYXK (冀) 2015-0038).

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