Colloids and Surfaces B: Biointerfaces 143 (2016) 415–422

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Electrospun tilapia collagen nanofibers accelerating wound healing via inducing keratinocytes proliferation and differentiation

Tian Zhou [a], Nanping Wang [b], Yang Xue [a], Tingting Ding [a], Xin Liu [a], Xiumei Mo [c,**], Jiao Sun [a,*]

[a] Shanghai Biomaterials Research & Testing Center, Shanghai Key Laboratory of Stomatology, Ninth People's Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200023, China
[b] Shanghai Fisheries Research Institute, Shanghai 200433, China
[c] College of Chemistry, Chemical Engineering and Biotechnology, Donghua University, Shanghai 201620, China

ARTICLE INFO

Article history:
Received 9 September 2015
Received in revised form 14 March 2016
Accepted 17 March 2016
Available online 18 March 2016

Keywords:
Electrospun tilapia collagen nanofibers
HaCaTs differentiation
Skin wound healing
Rat model

ABSTRACT

The development of biomaterials with the ability to induce skin wound healing is a great challenge in biomedicine. In this study, tilapia skin collagen sponge and electrospun nanofibers were developed for wound dressing. The collagen sponge was composed of at least two α-peptides. It did not change the number of spleen-derived lymphocytes in BALB/c mice, the ratio of $CD4^+/CD8^+$ lymphocytes, and the level of IgG or IgM in Sprague-Dawley rats. The tensile strength and contact angle of collagen nanofibers were 6.72 ± 0.44 MPa and $26.71 \pm 4.88^\circ$, respectively. They also had good thermal stability and swelling property. Furthermore, the nanofibers could significantly promote the proliferation of human keratinocytes (HaCaTs) and stimulate epidermal differentiation through the up-regulated gene expression of involucrin, filaggrin, and type I transglutaminase in HaCaTs. The collagen nanofibers could also facilitate rat skin regeneration. In the present study, electrospun biomimetic tilapia skin collagen nanofibers were successfully prepared, were proved to have good bioactivity and could accelerate rat wound healing rapidly and effectively. These biological effects might be attributed to the biomimic extracellular matrix structure and the multiple amino acids of the collagen nanofibers. Therefore, the cost-efficient tilapia collagen nanofibers could be used as novel wound dressing, meanwhile effectively avoiding the risk of transmitting animal disease in the future clinical apllication.

© 2016 Elsevier B.V. All rights reserved.

1. Introduction

Wound healing is a multifactorial process including inflammatory reaction, re-epithelialization, collagen deposition and angiogenesis. In order to protect the wound area and accelerate injured skin regeneration, safe wound dressings are expected to have excellent biological properties and the ability to induce tissue regeneration. It has been demonstrated in numerous studies that mammal collagen (especially porcine or bovine collagen) has excellent biocompatibility [1–3]. Therefore, this collagen has been extensively used for skin wound healing. However, mammal collagen still presents a risk of transmitting animal diseases, such as bovine spongiform encephalopathy and foot-and-mouth disease [4,5]. Additionally, the application of mammal collagen is restricted because of religious reason [6,7]. Recently, marine collagen has gradually attracted attention because of its abundance and low price. In 2008, Sankar et al. prepared collagen sheet from discarded fish scales (*Lates calcarifer*) that had enough tensile strength (2 MPa) for use as a wound dressing material [8]. Besides, fish collagen compounded with elastin or plant extracts had good biocompatibility with skin cells [4,9,10]. However, the immunogenicity of fish collagen and its biological functions for skin regeneration are still unknown. Thus, of great significance is to study these issues which are directly associated with future clinical applications of fish collagen as a wound-dressing material.

In recent years, studies focused on marine collagen have indicated that the denaturation temperature of tilapia fish scale collagen hydrogels reached 48 °C and increased to 62 °C after crosslinking [11], which was higher than body temperature and suggested good thermal stability. In 2012, Terada et al. constructed a chitosan-fish scale collagen composite scaffold and found that this scaffold was suitable for the attachment and proliferation of mucosal keratinocytes in an ex vivo-produced oral mucosa

* Corresponding author.
** Corresponding author.
E-mail addresses: xmm@dhu.edu.cn (X. Mo), jiaosun59@126.com (J. Sun).

http://dx.doi.org/10.1016/j.colsurfb.2016.03.052
0927-7765/© 2016 Elsevier B.V. All rights reserved.

equivalent [12]. Wang et al. also reported that deep-sea redfish skin had the highest contents of protein compared to scale and bone [13], which suggested that fish skin maybe optimal for the extraction of collagen and would have a high application value. In 2014, preliminary studies related to the biocompatibility of tilapia skin collagen were conducted by Yamamoto et al. [14]. However, it is unclear whether tilapia skin collagen could be prepared as a wound dressing with excellent biological effects to induce skin regeneration effectively.

It was known that human keratinocytes (HaCaTs) are one of the key cells involved in skin wound healing. The proliferation and differentiation of keratinocytes play important roles in wound re-epithelialization. Therefore, it is of great importance to study the biological effects of wound dressing on human keratinocytes and validate its healing effects in vivo, which are crucial to assess the potential of new materials for clinical application. Besides, biomimetic environment was also requried for tissue regeneration. Nanofibers fabricated by eletrospinning have adjustable diameters and mimic the structure and function of native extracellular matrix (ECM), which are beneficial for cell adhesion and proliferation. If tilapia skin collagen could be prepared as nanofibers by electrospinning, it might be helpful for its future application.

In the present study, high-purity fish collagen sponge was extracted from tilapia skin through a series of processing and purification technologies. Its amino acid composition and thermal denaturation temperature were analyzed, and the immunogenicity of collagen sponge was evaluated from the perspectives of humoral and cellular immunity. In addition, collagen nanofibers were further prepared by electrospinning. The morphological structure, tensile strength, thermal stability, swelling property and hydrophilicity of the collagen nanofibers were characterized. Furthermore, HaCaTs were chosen to investigate the effects of collagen nanofibers on promoting cell adhesion and proliferation. The mechanisms involved in collagen nanofibers inducing epithelium regeneration at the gene levels were investigated, focusing on the associations between HaCaTs differentiation and the expression of involucrin, filaggrin and type I transglutaminase. Finally, Sprague-Dawley (SD) rat models with full-thickness skin defects were used to confirm the ability of collagen nanofibers to accelerate wound healing. This research attempted to develop a novel biomimetic wound dressing with the function of inducing tissue regeneration, which also provides scientific basis for the future development and application of fish collagen in biomedicine.

2. Experimental

2.1. Preparation of tilapia collagen sponge

Tilapia skin (provided by Shanghai Fisheries Research Institute) was washed, chopped, and stirred in 0.1 M NaOH solution for 1–2 days. The samples were then soaked in 0.5–1 M acetic acid for 4–8 h with continuous stirring. The supernatant was collected by centrifugation and followed by the addition of 0.1–0.5% pepsin with stirring for 24–48 h. Next, 0.4 M ammonium sulfate was added and the precipitate was collected by centrifugation at 10,000g. The precipitate was then dissolved in 0.5–1 M acetic acid, dialyzed, and lyophilized to obtain collagen sponges for subsequent use. All procedures were carried out at 0–4 °C.

2.2. Characterization of tilapia collagen sponge

The purity and molecular weight of the tilapia collagen sponge were determined using sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) (GE Healthcare, SE 250, USA). The amino acid content of the collagen sponge was determined using Hitachi Amino Acid Analyzer (Hitachi, Japan). The denaturation temperature of the collagen sponge dissolved in 0.1 M acetic acid solution was measured with differential scanning calorimetry (DSC) (204 F1, Netzsch, Germany).

2.3. Lymphocyte proliferation assay

The experimental protocol was approved by the Animal Care and Experiment Committee of the Ninth People's Hospital affiliated to the School of Medicine, Shanghai Jiao Tong University. BALB/c mice were anesthetized using sodium pentobarbital and the spleens were collected under sterile conditions. The lymphocyte suspension was collected by adding red blood cell lysis buffer and diluting to 1×10^6 cells/mL. Lymphocytes were seeded on tilapia collagen sponges in 24-well plates. They were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin. In addition, lymphocytes seeded on cover slips without collagen sponge were used as negative control. Lymphocytes seeded on cover slips with the addition of concanavalin A (ConA) were used as positive control. After culture for 7 d, Cell Counting Kit-8 (CCK-8) solution was added, and the lymphocytes were further incubated for 3 h. The optical density (OD) values at 570 nm were measured using a spectrophotometer.

2.4. Subcutaneous implantation assay in SD rats

A total of eight healthy male 6–8-week-old SD rats (200–250 g) were used in this study. The experimental protocol was approved by the Animal Care and Experiment Committee of the Ninth People's Hospital affiliated to the School of Medicine, Shanghai Jiao Tong University. Eight rats were randomly divided into two groups. After anesthetization by sodium pentobarbital, the dorsal skin was longitudinally transected under sterile conditions. The subcutaneous tissues were separated, a tilapia collagen sponge with an area of approximately $1 \times 1\ cm^2$ was implanted, and the skin was then sutured. The control group received a sham operation without the implantation. Twenty-eight days after the operation, the SD rats were anesthetized, and blood samples were collected by cardiac puncture. The concentrations of IgG and IgM in the serum were detected using enzyme-linked immunosorbent assay (ELISA) reagent kits (Tbdscience, China), and $CD4^+$/$CD8^+$ T lymphocytes were counted by flow cytometry (FCM) (Guava easycyte, Millpore, Germany).

2.5. Fabrication and characteration of electrospinning tilapia collagen nanofibers

The tilapia collagen sponge was dissolved in a hexafluoroisopropanol (HFP) solution (Fluorochem Ltd, UK) with the weight concentration of 8%. The polymer was then placed in a plastic syringe and inserted in a syringe pump (789100C, Cole-Parmer, USA). The voltage and flow rate during electrospinning were 16–18 kV and 1.0 mL/h, respectively. The distance from the needle to the aluminum foil collector was 10–15 cm. The electrospun tilapia collagen nanofibers formed membranes and were further crosslinked using glutaraldehyde vapor and stored in a vacuum-drying oven. The morphology of the collagen nanofibers was observed using scanning electron microscopy (SEM) (JEOL JSM-5600, Japan). The mean fiber diameter and pore size were determined with image analysis software (Image-J, National Institutes of Health) and calculated by selecting 100 fibers or pore areas randomly. The chemical structure of tilapia collagen nanofibers was determined by Fourier transform infrared spectroscopy (FTIR) (Avatar 380, USA). The tensile strength was analyzed using a universal materials testing machine (H5K-S, Hounsfield, UK). The weight loss temperature was determined with a

Table 1
Real-time RT-PCR primer sets.

Gene/Oligo Name	Oligo Sequence
Involucrin forward	TCAATACCCATCAGGAGCAAATG
Involucrin reverse	GAGCTCGACAGGCACCTTCT
TGase1 forward	TCTTCAAGAACCCCCTTCCC
TGase1 reverse	TCTGTAACCCAGAGCCTTCGA
filaggrin forward	CCATCATGGATCTGCGTGG
filaggrin reverse	CACGAGAGGAAGTCTCTGCGT
GAPDH forward	TGAACGGGAAGCTCACTGG
GAPDH reverse	TCCACCACCCTGTTGCTGTA

thermogravimetric (TG) analyzer (209F1, Netzsch, Germany). The contact angle was measured using a contact angle measuring instrument (OCA40, Dataphysics, Germany). The surface area of the collagen nanofibers was measured using surface area analyzer (JWBK122T-B, China). The swelling property of the collagen nanofibers were studied by immersing the samples in PBS at 37 °C for 5 min, 1, 2, 3, 4, 8, 24, 48, 72, 96 and 120 h. Dry and wet weight of the collagen nanofibers were noted as *Wd* and *Ww*, respectively. Swelling ratio was determined by this equation:

$$\text{Swelling radio} = \frac{Ww - Wd}{Wd}$$

2.6. Cell adhesion and proliferation assay

HaCaTs (purchased from Kunming Animal Institute, China) were seeded on tilapia collagen nanofibers in 24-well plates, at a density of 2.5×10^4 cells/well in 500 μL of DMEM high-glucose medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin. After culture for 24 h, cell morphology was observed using SEM, fluorescence microscopy (Leica, Germany) and confocal laser-scanning microscopy (CLSM) (Carl Zeiss LSM 700, Germany). HaCaTs were then seeded on the collagen nanofibers at 2.5×10^4 cells/well, and HaCaTs seeded on cover slips made of high borosilicate glass without collagen nanofibers were used as a control. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed. After the HaCaTs were cultured for 1, 3 or 5 d, 5 mg/mL MTT (Sigma Chemical, St. Louis, Mo, USA) was added and the cells were incubated for 4 h. Dimethyl sulfoxide (DMSO) was then added to dissolve the crystals. The OD values at 570 and 630 nm were measured using a spectrophotometer (Labsystems Dragon Wellscan MK3, Finland). Cell viability was calculated according to the OD values.

2.7. Real-time polymerase chain reaction (PCR)

HaCaTs were seeded on tilapia collagen nanofibers at a density of 3×10^5 cells/well in 6-well plates. The cells seeded on cover slips were used as a control. After culturing for 24 h, total RNA was extracted using TRIZOL reagent (Invitrogen, USA). RNA (1.0 μg) was reverse-transcribed into cDNA using a PrimeScript first strand cDNA synthesis kit (TaKaRa, Japan). The expression of differentiation-related genes, such as involucrin, filaggrin, and type I transglutaminase (TGase1), was detected using real-time PCR with SYBR Premix EX Taq (TaKaRa, Japan) and a Bio-Rad sequence detection system (MyiQ2, USA). The qRT-PCR data were normalized to GAPDH values. The primers were listed in Table 1.

2.8. Skin wound healing experiment in SD rats

The experimental protocol was approved by the Animal Care and Experiment Committee of the Ninth People's Hospital affiliated to the School of Medicine, Shanghai Jiao Tong University. Twelve healthy 6–8-week old male SD rats with a body weight of 200–250 g (each) were injected with sodium pentobarbital. Three full-thickness skin defects with a diameter of 1.8 cm were incised on the dorsal region of each rat. These wounds were covered with tilapia collagen nanofibers or Kaltostat (ConvaTec) (n = 6), which was commonly used as wound dressing. The control group was covered without any material. Adhesive Tegaderm (3 M) polyurethane films were used to attach the dressings to the wounds. At 7 and 14 d after surgery, the morphology of the wounds was examined. Subsequently, the animals were sacrificed to collect skin tissues.

2.9. Histopathological examination

The harvested samples collected on days 7 and 14 were fixed in 10% formaldehyde for 1 week, paraffin-embedded, and sectioned into 4-μm-thick sections using a microtome (Leica, Germay). The sections were stained with hematoxylin-eosin (H&E) stain and then dehydrated, washed, mounted, and observed under a microscope.

2.10. Data analysis

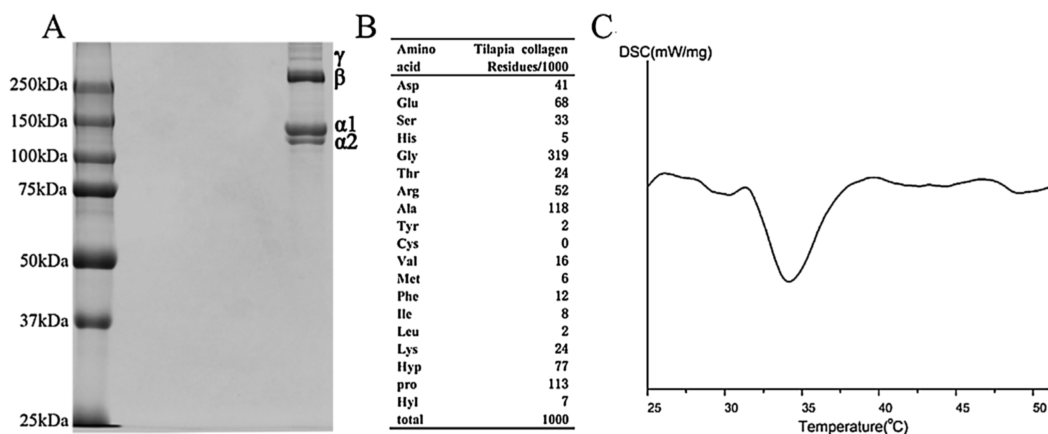
All the quantitative data were obtained at least in triplicate and expressed as the mean ± standard deviation (SD). Statistically significant differences ($p < 0.05$) among the different groups were evaluated using Student's t-test and one-way analysis of variance (ANOVA) with Tukey's post hoc multiple comparison test. All of the statistical analyses were performed using SPSS 11.0 software.

3. Results and discussion

In the present study, biomimetic electrospun fish collagen nanofibers were developed from tilapia skin through a series of extraction and purification techniques to consider the potential of using fish collagen as a biomaterial. The immunogenicity of tilapia collagen sponge, the cellular and histopathological effects of tilapia collagen nanofibers on wound healing were investigated. Additionally, whether the collagen nanofibers could be used as wound dressings were preliminarily assessed.

3.1. Characterization of tilapia collagen sponge

Currently, the commonly used technologies to extract and purify collagen include acid dissolution and pepsin digestion. The former can maintain the triple helix structure of collagen to a maximal extent, and the latter can reduce the antigenicity of collagen by removing the N-terminal and C-terminal regions of collagen peptides. We combined these two methods and exploited their individual advantages to obtain a tilapia collagen sponge. The electrophoresis patterns (Fig. 1A) showed that the extracted collagen sponge was mainly composed of α-chains (α1 and α2) and cross-linked chains (β and γ). The molecular weights of these chains were approximately 132 kDa (α1), 119 kDa (α2), 278 kDa (β) and greater than 300 kDa (γ), respectively. The electrophoretic migration rates and composition of chains suggested that the collagen sponge maintained its structural integrity, contained scarce amounts of impure proteins of low molecular weight, and had the typical type I collagen structure. To further analyze the purity of tilapia collagen, the amino acid composition of the collagen sponge was measured. The results showed that the collagen sponge contained 19 different amino acids (Fig. 1B). Glycine (Gly) was the most abundant (31.9%), and hydroxyproline (Hyp) and proline (Pro) accounted for 7.7% and 11.3%, respectively. Thus, the tilapia collagen sponge was consistent with type I collagen and was extracted with high purity. Because the hydrogen bonds formed by Hyp and Pro play a key role in maintaining the triple-helix structure of collagen, the content of these two kinds of amino acids has a close correlation with the denaturation temperature of collagen, which is a crucial index for evaluating



Amino acid	Tilapia collagen Residues/1000
Asp	41
Glu	68
Ser	33
His	5
Gly	319
Thr	24
Arg	52
Ala	118
Tyr	2
Cys	0
Val	16
Met	6
Phe	12
Ile	8
Leu	2
Lys	24
Hyp	77
pro	113
Hyl	7
total	1000

Fig. 1. Characterization of tilapia collagen sponge. (A) SDS−PAGE pattern. (B) Amino acid composition. (C) Thermal denaturation curve.

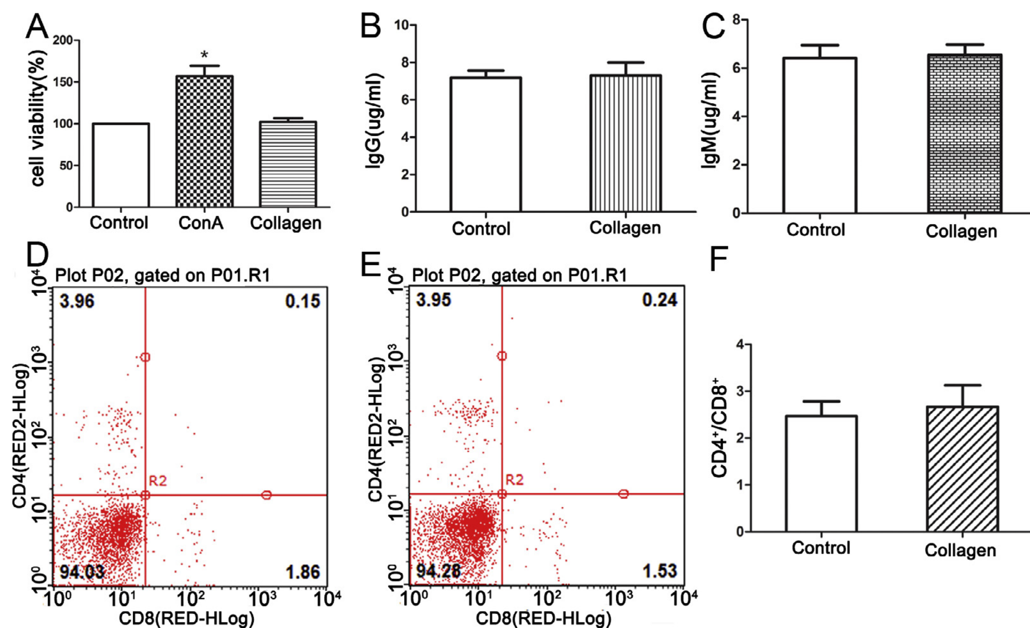


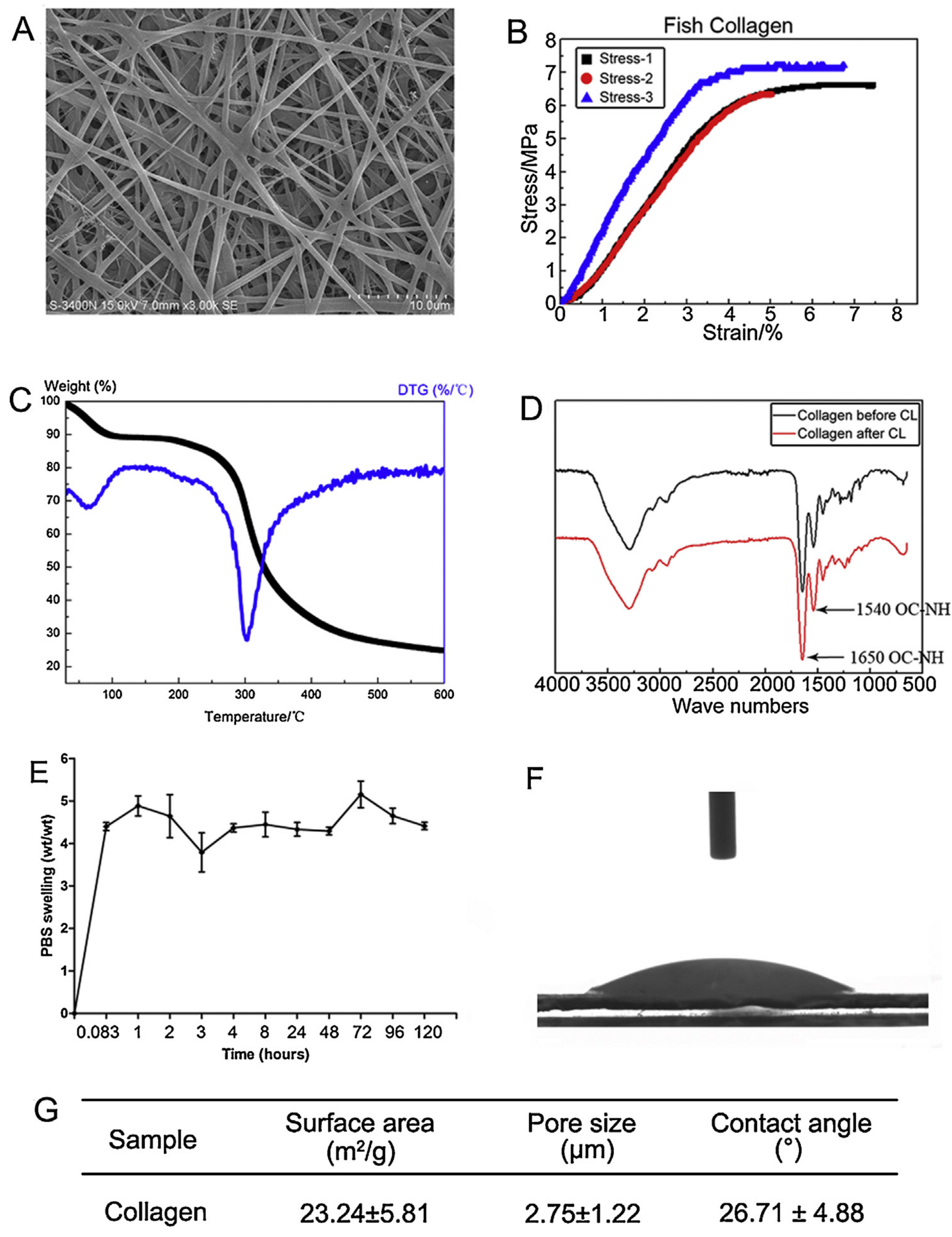
Fig. 2. Immunogenicity analysis of tilapia collagen sponge group and control group. (A) Proliferation of lymphocytes cultured on the collagen sponge for 7 days. Lymphocytes seeded on cover slips were used as a control. (B) The level of IgG in the serum of rats over 28 d. (C) The level of IgM in the serum of rats over 28 d. (D) Flow cytometry analysis of the control group. (E) Flow cytometry analysis of the collagen group. (F) The ratio of $CD4^+/CD8^+$ T lymphocytes. (B-F) The rats received sham operations were used as a control.

the thermal stability of collagen [15]. It was shown by DSC that the denaturation temperature of tilapia skin collagen sponge was 33.99 °C (Fig. 1C), which was close to that of tilapia scale collagen (36 °C) reported by Ikoma et al. [11,16]. These features indicated that tilapia collagen sponge had the potential for medical applications and provided the basis for further developing tilapia collagen nanofibers.

3.2. Immunogenicity of tilapia collagen sponge

Although tilapia collagen has an appropriate denaturation temperature for application in human, its immunogenicity as a heterogenic protein should also be paid much attention. Previous report has found that bovine collagen existed some immunogenicity and porcine collagen implants might cause allergic reactions and the elevation of IgG [17]. Whether tilapia collagen has immunogenicity is still unclear and has never been confirmed. The spleen is the largest immune organ in the body and contains a large number of immunocompetent B and T lymphocytes, which have the ability to recognize antigens and mediate humoral and cellular immunity, thus playing a primary role in immune response. Therefore, in this study, mixed lymphocytes were extracted from mouse spleen to evaluate the immunogenicity of tilapia collagen sponge in vitro. The results showed that the collagen sponge did not induce the proliferation of lymphocytes (Fig. 2A).

Furthermore, tilapia collagen sponge was implanted into the subcutaneous tissue of rats to investigate the immune response caused by the collagen sponge in the original state and small molecules produced during the process of its degradation. The two major antibodies, IgG and IgM, which play important roles in humoral immunity, were detected after 28 days. It was found that the collagen sponge did not induce significant changes in the level of IgG or IgM (Fig. 2B,C). Additionally, the ratio of $CD4^+$ and $CD8^+$ T lymphocytes was also evaluated. $CD4^+$ T lymphocytes participate in antigen recognition and signal transduction, whereas $CD8^+$ T lymphocytes kill infected cells. A relatively stable ratio of $CD4^+$ and $CD8^+$ T lymphocytes is important for coordinating cellular immunity. The results showed that the ratio of $CD4^+/CD8^+$ T lymphocytes in the collagen sponge group was similar to that of the control group



Sample	Surface area (m^2/g)	Pore size (μm)	Contact angle (°)
Collagen	23.24±5.81	2.75±1.22	26.71 ± 4.88

Fig. 3. Characterization of tilapia collagen nanofibers. (A) SEM images. (B) Stress/strain curves. (C) TG spectra. (D) FTIR spectra before and after cross-linking. (E) PBS swelling ratio. (F) Contact angle. (G) Surface area, pore size and contact angle.

(Fig. 2D–F). These finding indicated that the collagen sponge did not induce obvious immune response.

3.3. Characterization of electrospun tilapia collagen nanofibers

On the basis of the results that the tilapia collagen sponge did not generate immune responses, biomimetic wound dressings were chosen to be developed in this study. ECM is mainly composed of collagen and has a reticular structure. In order to biomimic ECM, tilapia collagen nanofibers were fabricated as membranes by electrospinning, which was a suitable technique for the production of small diameter fibers [18,19]. The nanofibers were further crosslinked using glutaraldehyde vapor. SEM showed that the collagen nanofibers were smooth with a diameter of 310 ± 117 nm (Fig. 3A), as it was reported that electrospun nanofibers could resemble the native topographical features of the natural ECM [20]. Because the biomechanical strength and thermal stability of collagen could be effectively enhanced by cross-linking [21,22], the tensile strength of the collagen nanofibers was analyzed using a universal material testing machine. The results showed that the tensile strength of the collagen nanofibers was 6.72 ± 0.44 MPa (Fig. 3B), which met the requirements for human skin. TG analysis and the first derivate related to the rate of weight loss (DTG) showed that there were two-stage sample destruction with the increase of temperature (Fig. 3C). The first stage from about 40 to 130 °C was associated with gradual loss of water. The second stage from about 190 to 600 °C was related to the weight loss of collagen. Such features indicated that the thermal stability of tilapia collagen nanofibers was suitable for human applications. These parameters might be influenced via the increased interaction between collagen molecules induced by cross-linking. It was because that cross-linking could stabilize the triple-helix structure of collagen, which enhanced the mechanical strength and thermal stability of the collagen nanofibers. To further determine whether

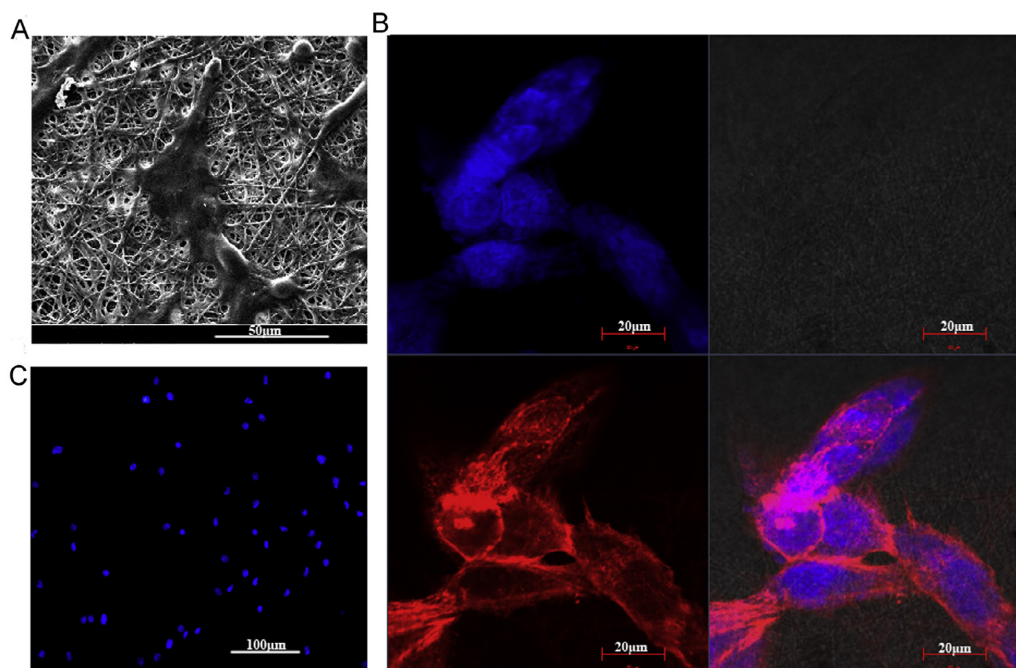


Fig. 4. Adhesion of HaCaTs cultured on tilapia collagen nanofibers. (A) SEM images of HaCaTs cultured for 1 day. (B) Fluorescence microscopy photographs of HaCaTs cultured for 1 day. The HaCaTs were stained with DAPI for nuclei (blue). (C) Confocal images of HaCaTs cultured for 1 day. The HaCaTs were double-stained with rhodamine-conjugated phalloidin for F-actin (red) and DAPI for nuclei (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

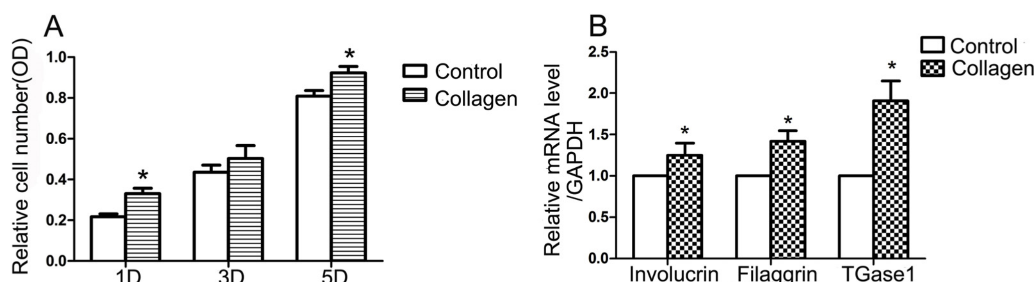


Fig. 5. Proliferation and differentiation of HaCaTs cultured on tilapia collagen nanofibers. (A) Proliferatio of HaCaTs cultured for 1, 3, and 5 days. (B) Differentiation-related genes expression of HaCaTs cultured on tilapia collagen nanofibers for 1 day. (A, B) HaCaTs seeded on cover slips were used as a control.

the chemical structure of the collagen nanofibers was altered after cross-linking, FTIR was performed to analyze the changes in the characteristic absorption peaks of the amide groups after cross-linking. It was found that the collagen nanofibers maintained α-helical structures after cross-linking (Fig. 3D).

3.4. Adhesion and proliferation of HaCaTs on tilapia collagen nanofibers

It was further investigated whether HaCaTs would be affected by tilapia collagen nanofibers with biomimetic ECM structure. Firstly, the effects of the collagen nanofibers on the adhesion and proliferation of HaCaTs were studied in vitro. The results illustrated that after HaCaTs were seeded on the collagen nanofibers for 24 h, the cells were firmly attached and equally distributed. They were also cross-linked with excellent morphology (Fig. 4). After being cultured on the collagen nanofibers for 5 d, the proliferation rate of the HaCaTs was 114% (Fig. 5A), indicating that the collagen nanofibers promoted cell adhesion and proliferation. These results might be explained by the nanostructure (Fig. 3A) and high specific surface area of collagen nanofibers (Fig. 3G). It was reported that a greater number of cells adhered on the nanofibers than that on the microfibers [23]. Scaffolds with biomimetic ECM structure and high specific surface area were more conducive to cell growth. Moreover, materials with high water absorption feature were optimal for cells to culture in. Fig. 3E showed that the collagen nanofibers presented a swelling ratio of approximately 4 fold compared with dry weight sample and the swelling ratio was highly increased at 5 min. This high swelling property is mainly due to hydrophilic nature of the material [24]. The result showed that the contact angle of the collagen nanofibers was $26.71 \pm 4.88^{\circ}$ (Fig. 3F,G), which indicated good hydrophilicity. It was because collagen had abundant hydrophilic groups like hydroxyl, carboxyl and amino groups. Due to these features, the collagen nanofibers were suitable for cell growth.

3.5. Epidermal differentiation induced by tilapia collagen nanofibers

In addition to confirming the ability of tilapia collagen nanofibers to promote adhesion and proliferation of HaCaTs during wound healing, it is also important to study the differentiation of epidermal keratinocytes which reveals the speed and quality of re-epithelialization. Normal epidermis should include basal layer, spinous layer, granular layer and cornified layer. Whether

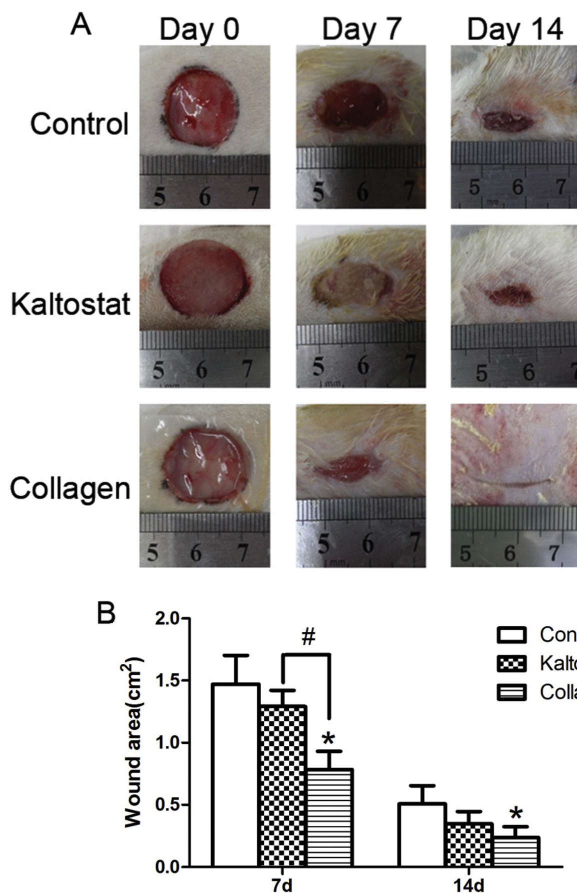


Fig. 6. Wound healing in SD rats. (A) The representative images of skin wounds after treatment with tilapia collagen nanofibers or Kaltostat, with untreated wounds as control (n = 6). (B) Wound areas at different time points after treatment.

collagen nanofibers can induce keratinocyte differentiation to each layer is still unknown. Therefore, keratinocyte (HaCaTs) differentiation influenced by collagen nanofibers was investigated without the addition of any growth factors. It was found that the collagen nanofibers significantly upregulated the expression of involucrin, filaggrin, and TGase1 genes (Fig. 5B). Involucrin and filaggrin were specifically expressed in suprabasal epidermal layers. TGase1 is a key enzyme in the synthesis of cornified envelopes, which indicates the formation of cornified layer. The results demonstrated that the collagen nanofibers induced keratinocyte differentiation, which is required for the formation of integrated epidermis. The possible mechanism may involve the synergetic effects of the amino acids on stimulating the differentiation of HaCaTs. As shown in Fig. 1B, collagen sponge contained significant amounts of Pro. It has been reported that Pro may play an important role in maintaining cell structure and function, as well as wound healing [25]. Our previous studies also found that the hydrolyzed tilapia collagen could induce cell differentiation which might be related to its variety of amino acids [26]. These data further confirmed that the amino acids of tilapia collagen nanofibers might be conductive to HaCaTs differentiation.

3.6. Skin regeneration induced by tilapia collagen nanofibers

Ultimately, to validate the effects of tilapia collagen nanofibers on wound healing, dorsal full-thickness skin-defect models in SD rats were designed. Compared to the control groups, the wound-healing rate was significantly improved, crust started to disappear at day 7, and most of the wound area was covered with a continuous

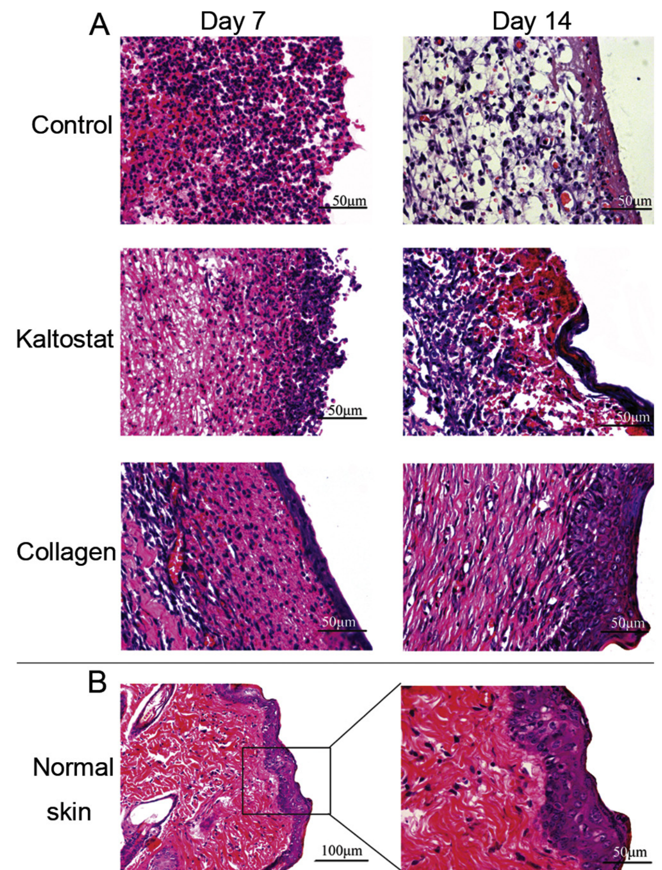


Fig. 7. Representative images of H&E staining. (A) Wound sections treated with tilapia collagen nanofibers or Kaltostat, with untreated wounds as control group, at days 7 and 14. (B) Normal skin.

epidermis at day 14 in the collagen nanofibers group (Fig. 6), while the skin wounds in the other two groups were not fully healed. The histopathological results confirmed that the collagen nanofibers caused the lowest degree of inflammatory response and induced the best growth status of new epidermis throughout the process of wound healing (Fig. 7). The inflammatory response was significantly reduced at day 7, and new epidermis with intact structure and good continuity could also been found at day 14. The epidermal cells were fully differentiated, basal cells were closely arranged, the horny layer could be observed, and layers of keratinocytes were evident. The above results indicated that collagen nanofibers could accelerate wound healing by promoting re-epithelialization, which could be explained by the results of experiments in vitro. The collagen nanofibers developed in our study could effectively promote wound healing without generating immune responses. Therefore, the in vivo findings provided the essential theoretical basis for great potential of collagen nanofibers used in tissue regeneration applications.

4. Conclusion

In the present study, tilapia skin collagen sponge and the original electrospun collagen nanofibers were successfully fabricated respectively. The collagen sponge elicited no immune responses. The tensile strength and thermal stability of the collagen nanofibers were suitable for application on human skin. What's more, the collagen nanofibers could promote the adhesion, proliferation and differentiation of HaCaTs with enhanced expression of involucrin, filaggrin and TGase1. It was further confirmed that the collagen nanofibers significantly stimulated reepithelialization in wound

healing of the rat model. These effects were probably owed to the biomimetic structure and multiple amino acids of the collagen nanofibers. The study indicated that the novel biomimetic electrospun tilapia skin collagen nanofibers could accelerate wound healing rapidly and effectively without inducing any immune responses, thus providing a possibility for the future application of tilapia collagen nanofibers in skin regeneration.

Contributors

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Funding

The authors declare no competing financial interests.

Acknowledgment

This work was supported by grants from Natural Science Foundation of China (nos. 31470917, 31470941).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.colsurfb.2016.03.052.

References

[1] A.L. Parcells, J. Karcich, M.S. Granick, M.A. Marano, Eplasty 14 (2014) e36.
[2] L. Ding, X. Li, H. Sun, J. Su, N. Lin, B. Péault, T. Song, J. Yang, J. Dai, Y. Hu, Biomaterials 35 (2014) 4888–4900.
[3] A.M. Pabst, K.M. Lehmann, C. Walter, M. Krüger, S.I. Stratul, A. Kasaj, Odontology 104 (2016) 19–26.
[4] Y. Matsumoto, K. Ikeda, Y. Yamaya, K. Yamashita, T. Saito, Y. Hoshino, T. Koga, H. Enari, S. Suto, T. Yotsuyanagi, Biomed. Res. 32 (2011) 29–36.
[5] Y. Zhang, W. Liu, G. Li, B. Shi, Y. Miao, X. Wu, Food Chem. 103 (2007) 906–912.
[6] M.C. Gómez-Guillén, B. Giménez, M.E. López-Caballero, M.P. Montero, Food Hydrocoll. 25 (2011) 1813–1827.
[7] R. Duan, J. Zhang, X. Du, X. Yao, K. Konno, Food Chem. 112 (2009) 702–706.
[8] S. Sankar, S. Sekar, R. Mohan, S. Rani, J. Sundaraseelan, T.P. Sastry, Int. J. Biol. Macromol. 42 (2008) 6–9.
[9] T. Muthukumar, P. Prabu, K. Ghosh, T.P. Sastry, Colloids Surf. B 113 (2014) 207–212.
[10] T. Muthukumar, R. Senthil, T.P. Sastry, Colloids Surf. B 102 (2013) 694–699.
[11] S. Chen, N. Hirota, M. Okuda, M. Takeguchi, H. Kobayashi, N. Hanagata, T. Ikoma, Acta Biomater. 7 (2011) 644–652.
[12] M. Terada, K. Izumi, H. Ohnuki, T. Saito, H. Kato, M. Yamamoto, Y. Kawano, K. Nozawa-Inoue, H. Kashiwazaki, T. Ikoma, J. Tanaka, T. Maeda, J. Biomed. Mater. Res. B Appl. Biomater. 100 (2012) 1792–1802.
[13] L. Wang, X. An, Z. Xin, L. Zhao, Q. Hu, J. Food Sci. 72 (2007) E450–455.
[14] K. Yamamoto, K. Igawa, K. Sugimoto, Y. Yoshizawa, K. Yanagiguchi, T. Ikeda, S. Yamada, Y. Hayashi, BioMed Res. Int. 2014 (2014) 630757.
[15] T. Minh Thuy le, E. Okazaki, K. Osako, Food Chem. 149 (2014) 264–270.
[16] T. Ikoma, H. Kobayashi, J. Tanaka, D. Walsh, S. Mann, Int. J. Biol. Macromol. 32 (2003) 199–204.
[17] S.H. Bentkover, Facial Plast. Surg. 25 (2009) 73–85.
[18] A. Yin, K. Zhang, M.J. McClure, C. Huang, J. Wu, J. Fang, X. Mo, G.L. Bowlin, S.S. Al-Deyab, M. El-Newehy, J. Biomed. Mater. Res. A 105 (2013) 1292–1301.
[19] L. Kolacna, J. Bakesova, F. Varga, E. Kostakova, L. Planka, A. Necas, D. Lukas, E. Amler, V. Pelouch, Physiol. Res. 56 (2007) S51–S60.
[20] H. Duan, B. Feng, X. Guo, J. Wang, L. Zhao, G. Zhou, W. Liu, Y. Cao, W.J. Zhang, Int. J. Nanomed. 8 (2013) 2077–2084.
[21] G. Wollensak, E. Spoerl, J. Cataract Refract. Surg. 30 (2004) 689–695.
[22] Z. Tian, K. Wu, W. Liu, L. Shen, G. Li, Spectrochim. Acta A 140 (2015) 356–363.
[23] F. Tian, H. Hosseinkhani, M. Hosseinkhani, A. Khademhosseini, Y. Yokoyama, G.G. Estrada, H. Kobayashi, J. Biomed. Mater. Res. A 84 (2008) 291–299.
[24] D. Archana, J. Dutta, P.K. Dutta, Int. J. Biol. Macromol. 57 (2013) 193–203.
[25] G. Wu, F.W. Bazer, R.C. Burghardt, G.A. Johnson, S.W. Kim, D.A. Knabe, P. Li, X. Li, J.R. McKnight, M.C. Satterfield, T.E. Spencer, Amino Acids 40 (2011) 1053–1063.
[26] C. Liu, J. Sun, Biomacromolecules 15 (2014) 436–443.