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Evaluation of biocompatibility and immunogenicity of micro/nanofiber materials based on tilapia skin collagen

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Abstract

Type I collagen, used as a raw material, plays a pivotal role in the development of medical devices and tissue engineering. Due to the risk of zoonotic transmission and religious constraints for mammalian collagen, fish collagen gains increased attention and is widely seen as an alternative. In this study, two collagen micro/nanofiber materials, self-assembled collagen nanofiber and electrospun collagen nanofiber, were prepared by tilapia skin collagen and their biocompatibility and immunogenicity was thoroughly investigated. The result revealed that the state of tilapia skin collagen in self-assembled collagen nanofiber and electrospun collagen nanofiber was different. The circular dichroism spectrum indicated that collagen in self-assembled collagen nanofiber retained the triple helical structure of the native collagen, while collagen in electrospun collagen nanofiber was denatured into gelatin. Nevertheless, the evaluation according to ISO10993, including tests of cytotoxicity, hemolysis, skin sensitization, acute systemic toxicity, mouse immunization and lymphocyte proliferation, demonstrated good biocompatibility and low immunogenicity for both self-assembled and electrospun collagen nanofiber materials. Overall, the present study highlighted that type I collagen from tilapia skin would be a promising biomaterial for the development of regenerate medical products.

Keywords

Tilapia skin collagen, micro/nanofiber, biocompatibility, immunogenicity, biosafety

Introduction

Type I collagen is the main structural protein in the extracellular matrix of animal tissues,[1] which has been proven to be safe and effective for its low immunogenicity, good biocompatibility and biodegradability.[2–4] So it has been processed into diverse medical products such as powder, sponge, fiber and hydrogel, and applied in clinical indications.[5–8] Currently, the main source of medical collagen is the mammalian connective tissues.[9] Mammalian collagen raises several concerns, including the limited source, high cost and potential risk of zoonotic transmission as well as religious restriction in Islamism and Muslim communities. In view of these limitations of mammalian collagen, fish collagen is seen as a promising alternative.[10] Type I collagen of marine fishes is well characterized, such as that of catfish, seaweed pipefish, pufferfish and tuna.[11,12] Considerable work is also done on collagen from fresh water fish, including silver carp, rohu and catla.[13,14]

More recently, tilapia skin collagen (TSC) is attracting increasing attention. As compared with other fish species, the collagen from tilapia has its advantages. Tilapia skin contains abundant high quality collagen, and is widely available as a by-product. Tilapia are raised in aquaculture farms at huge industrial scale in

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many countries including China,[15] which enables the establishment of the source material traceability system for risk management and regulatory supervision of medical products. More importantly, TSC is more stable than the collagen from marine fish species.[16] Based on the above benefits, TSC was prepared to various biomedical products including sponge for hemostatic material[17] and skin regeneration,[18] electrospun nanofibers for guided bone regeneration.[19] However, these studies mainly focused on the effectiveness of TSC rather than its biosafety. It is conceivable that collagen extraction and other manufacturing processes may change the state of collagen structure and its biosafety.[20,21]

Self-assembly and electrospinning are two preparation methods for collagen fibers. Under suitable conditions, collagen molecules will self-assemble into microfibers for application.[22,23] While, electrospinning is also a cost-effective and simple technique for producing continuous nanofiber, which uses a strong electric field to a pumped collagen solution to induce its flight toward a collector whereas the solvent is being evaporated.[24] But few papers revealed if the collagen had changed in the two different processing procedures. In addition, immunogenicity was a critical factor for the biological safety of collagen.[25] Earlier studies of TSC were primarily on cytocompatibility, sensitization or subcutaneous implantation, while few on systematic evaluation for the immunogenicity.

Therefore, these two types of micro/nanofibers, self-assembled collagen fiber (S-CNF) and electrospun collagen fiber (E-CNF), were fabricated from TSC in this paper, and the state of collagen in the two fibers was characterized by circular dichroism (CD) spectra and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The biocompatibility and immunogenicity of S-CNF and E-CNF were extensively evaluated according to ISO10993, including tests of cytotoxicity, sensitization test, hemolysis test, acute systemic toxicity and immunogenicity. Thus, the aim of this study is to discuss the biocompatibility and immunogenicity of TSC in different state.

Materials and methods

Materials

Purified TSC was a gift by Shandong International Biotechnology Park Development Co., Ltd. The extraction and purification of this collagen material were detailed in the Chinese patent (CN201410797201.9). A commercial bovine collagen sponge (Kejibang®,Wuxi Biot Biology Technology Co., Ltd) was used as the control material for comparison.

Determination of non-collagenous proteins in TSC

The non-collagenous proteins content in TSC was determined using SDS-PAGE with 8% resolving gel and 4% stacking gel according to YY0954-2015 (a Chinese industry standard for medical material) with minor modification. The limit of detection by SDS-PAGE method was first investigated by using a serial diminishing bovine serum albumin (BSA) amounts (250 ng, 125 ng, 50 ng, 25 ng, 20 ng, 10 ng and 5 ng). The intensity of electrophoresis gel bands (gray scale) was detected by quantitative software for SDS-PAGE.

TSC samples were dissolved in 0.5 M acetic acid to 1 mg/mL and freeze-dried for 48 hours to prepare collagen samples with greater water solubility. The freeze-dried sample was then dissolved in water to 10 mg/mL that was in turn diluted by the volume ratio 9:1 with a 20 U/mL collagenase I solution. After incubation at 37°C for 4 hours, the remaining non-collagenous protein was detected by the SDS-PAGE. As control groups, the collagen solution without digestion and the collagenase I solution were analyzed simultaneously. The intensity of electrophoresis gel bands was detected after staining and decolorization. The non-collagenous proteins content was calculated as follows:

When B-C $\neq 0$, calculated according to formula (1):

$$\text{The purity of collagen in sample } (\%) = \mathrm{A} - (\mathrm{B} - \mathrm{C}) \quad (1)$$

When B-C $= 0$, calculated according to formula (2):

$$\text{The purity of collagen in sample } (\%) = (10{,}000 - \mathrm{D})/10{,}000 \times 100\% \quad (2)$$

$$\text{The non collagenous protein content } (\%) = 100\% - \text{The purity of collagen in sample } (\%) \quad (3)$$

where A is the band intensity of the TSC solution; B is the band intensity of the digested TSC solution; C is the band intensity of the collagenase solution; D is the limit of detection for BSA in the test (ng).

Preparation of S-CNF

TSC was dissolved in 0.5 M acetic acid to 5 mg/mL collagen solution at 4°C. This collagen solution was diluted by PBS (pH 7.4) at 1:10 (v/v) and left to stand at 30°C over night for collagen micro/nanofibers formation via self-assembly. The solution with micro/nanofibers was stirred and then filtered through a 100-mesh sieve. The collagen on the sieve was reconstituted in water at 1:5 (w/w). Finally, S-CNF was prepared by

freeze-drying for 48 hours and sterilization with 15 kGy Co-60 γ-ray irradiation for following experimental study.

Preparation of E-CNF

The mechanical property of E-CNFs is known to be very poor. To facilitate the process, electrospun PLGA fibers were prepared simultaneously, and were combined with pure collagen fibers in the E-CNF preparation. The process was as follows: 2.0 g TSC and 2.0 g PLGA (Jinan Daigang Biomaterial Co., Ltd, China) were dissolved separately in 20 mL hexafluoroisopropanol. Then the two solutions were loaded into two syringes (with inner diameter 0.25 mm) placed on the opposite sides of the collector. The distance between the tip of the syringe and the collector was 15 cm. The electrospinning voltage applied to the two needles was 15 kV, and the pump flow rate was set at 1 mL/hours. The collected membrane was vacuum-dried for 48 hours after the spinning process. E-CNF was also sterilized with 15 kGy Co-60 γ ray irradiation for the following experimental study.

Characterization of S-CNF and E-CNF

Scanning electron microscopy (SEM). The morphology of S-CNF and E-CNF was examined using a scanning electron microscope (SEM Zeiss, EVO LS15, Germany) in Binzhou Medical University. Samples were gold-coated in a sputter coater and observed at an acceleration voltage of 10 kV by EVO LS15 SEM at 200× to 5000× magnification.

Circular dichroism spectra. S-CNF and E-CNF were dissolved in 0.5 M acetic acid for sample solution at a concentration of 0.1 mg/mL. After centrifugation at 12,000 r/min for 20 min at 4°C, the spectra of supernatants were detected from 180 to 260 nm (Chirascan, Applied Photophysics Ltd., UK) to identify whether the collagen was denatured.

SDS-PAGE chromatography. SDS-PAGE was used to investigate the subunit composition and molecular weights of collagen in S-CNF and E-CNF. S-CNF and E-CNF were dissolved in 0.1 M acetic acid at a concentration of 1 mg/mL and 2 mg/mL, respectively. SDS-PAGE was carried out with 8% resolving gel and 4% stacking gel. The molecular weights of the subunits were determined by the band's location.

Evaluation of biocompatibility

Cytotoxicity. The sample extracts were prepared specified to ISO 10993-12:2007. S-CNF or E-CNF was extracted using DMEM medium at 6 cm^2/mL for 24 hours at 37°C. The obtained solution was marked as "100%" extract solution, which was further diluted with DMEM to prepare 75%, 50% and 25% sample extract solutions. L929 cells were seeded into 96-well plate at a density of 1×10^4 cells/well and inoculated for 24 hours. The wells including L929 cells were divided into negative group, positive group, S-CNF test groups and E-CNF test groups. All the medium in wells were removed, a 100 μL aliquot of each sample solutions was added in the respective wells. The DMEM was used as blank control group. Phenol solution (0.05%, w/v) and the extract solution of high-density polyethylene were used as positive (toxic) and negative (non-toxic) control, respectively. The 96-well plates were inoculated for another 24 hours. Cell viability was determined by methylthiazol tetrazolium (MTT) method.

Hemolysis test. Sample extracts were prepared by soaking in normal saline at 6 cm^2/mL for 72 hours at 37°C. Blood samples were collected from rabbit into tubes coating with heparin. Then, the collected blood samples were diluted two times with normal saline. The experiment was carried out in four groups: the S-CNF test group, the E-CNF test group, normal saline group (negative control) and distilled water group (positive control). The extract solutions of S-CNF or E-CNF, normal saline and distilled water (all in 3 mL) were added to respective tubes and inoculated for 30 min at 37°C. The aliquots of 60 μL diluted blood were added into each tube and mixed gently. After 60 min inoculation, the solution tubes were centrifuged at $800 \times g$ for 5 min and the absorbance (Ab) of supernatant was detected at 545 nm. Hemolytic rate was calculated as follows:

$$\begin{aligned}&\text{Hemolytic rate } (\%)\\&= ((\text{Ab of test group} - \text{Ab of saline group})\\&\quad /(\text{Ab of water group} - \text{Ab of saline group})) \times 100\%\end{aligned} \tag{4}$$

Skin sensitization test. The Guinea Pig Maximization Test (GPMT)[19] was used to evaluate the sensitization of S-CNF and E-CNF. Sample extracts were prepared by soaking in the normal saline at 6 cm^2/mL for 72 hours at 37°C. Thirty albino guinea pigs (Beijing Vital River Laboratory Animal Technology Co., Ltd.) were randomly divided into four groups: 10 for S-CNF test group and E-CNF test group, and 5 for negative control group and positive control group. The animals in test groups were initially exposed to 0.1 mL/site of S-CNF or E-CNF extract

solution by intracutaneous injection into the back of the scapula, which were with or without Freund's complete adjuvant (FCA) in different sites. Seven days after induction, 10% (w/w) sodium dodecyl sulfate-paraffin solution was applied topically to the injected sites and wiped clean after 24 hours. Then patches impregnated with S-CNF or E-CNF extract solutions were applied onto the injected sites and removed after 48 hours. Fourteen days after induction, patches impregnated with S-CNF or E-CNF extract solutions were closely pasted on the abdomen for 24 hours to stimulate inflammatory response. The animals of negative control group and positive control group were injected intracutaneously with normal saline or 0.1% (w/w) 2,4-dinitrochlorobenzene similarly. The skin response was observed and graded at 24, 48 and 72 hours after stimulation according to the criteria proposed by Magnusson and Kligman.[20] Scores were judged as follows: 0 (no visible change), 1 (discrete or patchy erythema), 2 (moderate and confluent erythema), and 3 (severe erythema and swelling).

Acute systemic toxicity and immunization test

Mouse immunization and acute systemic toxicity test were integrated together to reducing the number of used animals on the base of general reference to ASTM F1906-1998(2003), ISO/TS 10993-20:2006 and ISO 10993-11:2006.

Animal model. BALB/c strain mice were used because of their susceptibility to collagen reported previously. As shown in Table 1, 60 female BALB/c strain mice about 8 weeks old were randomly divided into four groups: negative control group, bovine collagen sponge group (BOVINE), S-CNF group and E-CNF group. The samples were implanted subcutaneously by trocar in these groups. The animal model was simultaneously used for acute systemic toxicity and immunization tests.

Acute systemic toxicity. The animals were observed immediately after implantation. Additionally, the weight was recorded at 4, 24, 48, and 72 hours after implantation. The acute systemic toxicity was graded according to reaction and the rating criteria per ISO 10993-11:2006. The sample was affirmed to be compliance with the requirements if the reaction of experimental groups was not greater than that of the control group during observation. The sample was not in compliance with the requirements if any of the following happened: more than two animals died; more than two animals showed reactions associated with toxicity, such as convulsions and weakening; more than three animals showed a more than 10% weight loss.

Antibody analysis. The test bleeds were taken at 2, 4 and 8 weeks after implantation. The obtained blood samples were allowed to clot and centrifuged to separate and collect sera. All sera samples were diluted 160,000 times and then analyzed by an ELISA kit (ADI, USA) for mouse IgG antibodies.

Weight coefficient of immune organ. Animals were sacrificed after weighing and bleeding at 2, 4 and 8 weeks after implantation. Thymus and spleen were collected and weighed. Thymus coefficient and spleen coefficient were calculated using the follow formulas:

$$\text{Thymus coefficient (mg/g)} = \text{weight of thymus} \times 1000/\text{weight of mice} \quad (5)$$

$$\text{Spleen coefficient (mg/g)} = \text{weight of spleen} \times 1000/\text{weight of mice} \quad (6)$$

Lymphocyte proliferation test. Lymphocyte proliferation test was carried out according to ASTM F1906-1998 (2003) with minor modification. All the following experiments were carried out under sterile conditions. S-CNF or E-CNF was extracted using the Roswell Park Memorial Institute medium (RPMI 1640 medium, containing 10% fetal calf serum and 0.3 mg/mL glutamine) at 6 cm^2/mL for 72 hours at 37°C. Lymphocytes of BALB/c mice were isolated by differential gradient sedimentation. Isolated lymphocytes

Table 1. Immunological testing of collagen sample in BALB/c strain mice.

Group	Sample	Dose	Route
Negative control	Sterile PBS solution	0.1 mL	Subcutaneous
S-CNF	Self-assembled collagen nanofiber	2.2 mg	Subcutaneous
E-CNF	Electrospun collagen nanofiber	2.2 mg	Subcutaneous
BOVINE	Bovine collagen sponge	2.2 mg[a]	Subcutaneous

S-CNF: self-assembled collagen fiber; E-CNF: electrospun collagen fiber; BOVINE: bovine collagen sponge group.
[a]The weight of collagen sponge (120 mm × 40 mm × 3 mm) was about 0.1 g. Corresponding to the dosage of 0.5 g for an adult weighing 55 kg, a 2.2 mg dosage was derived for a 20 g mice according to the body surface area conversion formula of dosing calculation.

were suspended at 2×10^6 cells/mL in RPMI 1640 medium (negative group), RPMI 1640 medium with 5 μg/mL concanavalin A (ConA, positive group), S-CNF extract (S-CNF group) or E-CNF extract (E-CNF group), respectively. Aliquots of 200 μL of sample solution were added into 96-well plate and incubated at 37°C in 5% CO_2 for 3 days. Lymphocyte proliferation was examined by using the MTT method and the Ab was determined at 570 nm. The proliferation for S-CNF and E-CNF was compared to that of the negative group.

Statistical analysis

Quantitative data were expressed as mean ± standard deviation (SD). The differences between test groups were evaluated by independent samples t-test using SPSS 13.0 with the criterion of $p < 0.05$ being considered significantly different.

Result and discussion

Determination of non-collagenous proteins in collagen

Non-collagenous proteins were among the major inducers of immunogenicity in collagen-based medical products. In this study, the content of non-collagenous proteins in TSC was determined to exclude its interference with collagen. The purity of soluble collagen can be determined by SDS-PAGE after digestion with collagenase, which was mentioned in YY0954-2015 and ASTM F2212-2011. The collagen should be water soluble in the two standards; however, the water-solubility of TSC in our study was poor. So, the collagen sample was dissolved in water and then freeze-dried. The obtained collagen samples could be dissolved directly in water and used for the determination of non-collagenous proteins. As shown in Figure 1, the limit of detection was 25 ng for BSA, and the non-collagenous protein in TSC was examined. The intensity of electrophoresis gel bands of the digested TSC sample was faint, comparable to that of the collagenase solution used for digestion. The content of non-collagenous proteins of TSC, calculated according to formula (2) and formula (3), was 0.25%. Therefore, the purity of TSC was extremely high, and the interference of non-collagenous proteins would be negligible for the immunogenicity test of TSC in the present study.

Characterization of S-CNF and E-CNF

The S-CNF material exhibits as micro-fibers with a non-uniform diameter distribution, and the fiber diameters were 5.83 ± 5.79 μm. Moreover, some collagen fibers appear to self-assemble further into the flake-like structures. E-CNF was primarily nanofibers with relatively uniform diameter (Figure 2) and the fiber diameters were 639 ± 219 nm. The mechanical property of E-CNF was strong enough to withstand the forces during operation.

To determine whether the state of collagen structure was altered upon the process, CD spectra of S-CNF and E-CNF were compared. Native collagen molecules have an intact triple helical structure, and there are two distinguish absorption peaks at 221 nm and 198 nm in the CD spectrum of natural collagen.[26] If collagen state or structure changes, the triple helical structure would be disrupted, resulting in the alterations of the CD spectrum. In the denatured collagen, the positive peak at 221 nm is not present and the negative peak at 198 nm shifts toward the longer wavelength in the CD spectrum. As shown in Figure 3, the CD spectrum of S-CNF exhibited specific absorption peaks at 221 nm and 198 nm that are consistent with the native collagen. However, the spectrum of E-CNF lacks the positive peak at 221 nm, which indicated the triple helical structure

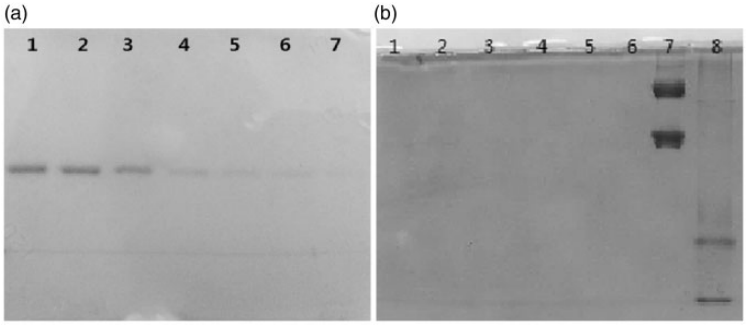

Figure 1. The SDS-PAGE result for evaluation of non-collagenous proteins in TSC. (A) The limit of detection, lanes 1–7: BSA 250 ng, 125 ng, 50 ng, 25 ng, 20 ng, 10 ng and 5 ng; (B) the determination of noncollagenous proteins in TSC, lanes 1–3: the collagenase solution, lanes 4–6: the digested solution of TSC, lane 7: the solution of TSC, lane 8: protein marker.
SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TSC: tilapia skin collagen.

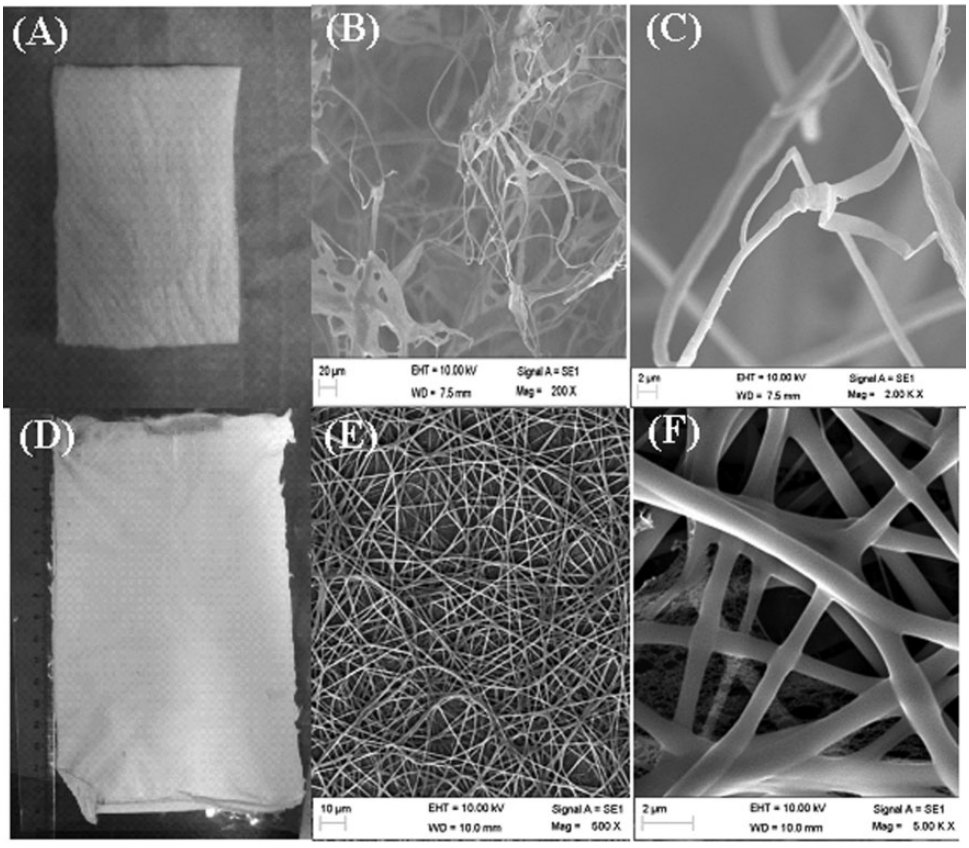

Figure 2. Typical photos and SEM micrographs of S-CNF and E-CNF. (A) Photos of S-CNF; (B) SEM images of S-CNF (200×); (C) SEM images of S-CNF (2000×); (D) photos of E-CNF; (E) SEM images of E-CNF (500×); (F) SEM images of E-CNF (5000×).
SEM: scanning electron microscopy; S-CNF: self-assembled collagen fiber; E-CNF: electrospun collagen fiber.

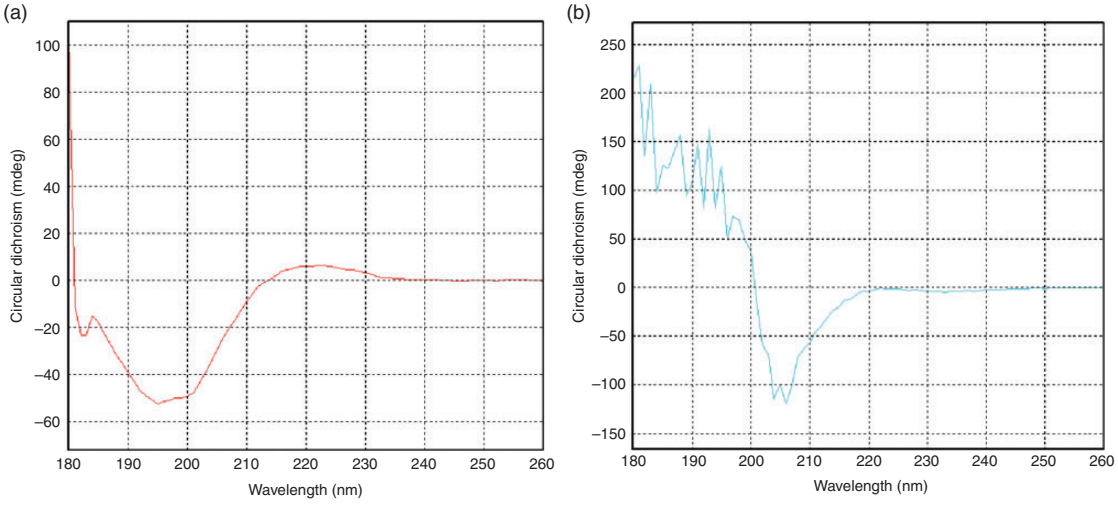

Figure 3. The circular dichroism spectra of S-CNF (A) and E-CNF (B).
S-CNF: self-assembled collagen fiber; E-CNF: electrospun collagen fiber.

was destroyed during the electrospinning. The triple helical structure of collagen in E-CNF was disrupted because of HFIP used in electrospinning process. HFIP, as an organic solvent, can make collagen denaturation by destroying hydrogen bond in collagen molecule, while hydrogen bond is an important factor to maintain the triple helical structure of collagen molecule.

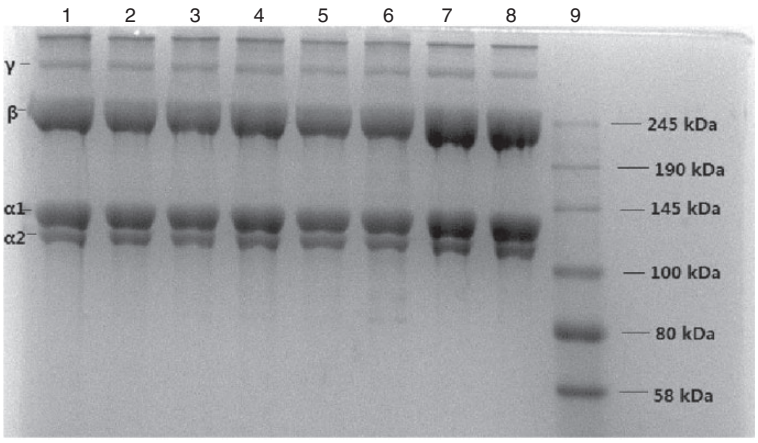

Figure 4. SDS-PAGE of S-CNF and E-CNF. Lanes 1 and 2: the solution of TSC; lanes 3 -5: S-CNF, lanes 6 -8: E-CNF; lane 9: protein marker.
SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; S-CNF: self-assembled collagen fiber; E-CNF: electrospun collagen fiber; TSC: tilapia skin collagen.

SDS-PAGE was used for measuring the subunit composition and molecular weights in S-CNF and E-CNF. The process effect on collagen molecular weights was studied by comparing with TSC material and bovine collagen. SDS-PAGE chromatography (Figure 4) proved that TSC had typical characteristic subunits of type I collagen including α1, α2, β and γ. In addition, SDS-PAGE chromatography of all test samples including E-CNF was similar, which demonstrated that the collagen in E-CNF was not fragmented into polypeptides of smaller molecules. Although CD spectrum showed the triple helical structure of collagen in E-CNF was destroyed, the denatured process mainly occurred among the triple helical structure. Therefore, the electrospinning process causes the gelatinization of TSC in E-CNF.

Biocompatibility evaluation

Cytotoxicity and hemolysis test. L929 cells were co-cultured with the S-CNF or E-CNF for evaluating cytotoxicity using the MTT assay. Compared to the blank control group, cell viability was from 94.7% to 113.8% for S-CNF and from 87.4% to 103.8% for E-CNF at different concentration as shown in Figure 5. According to ISO 10993-5:2009, the cytotoxicity of S-CNF was at level 1, whereas E-CNF was at level 2. The difference was speculated to be hexafluoroisopropanol residue used in the electrospinning process. The residual solvent resulted in some toxicity, demonstrating that the electrospinning process should be optimized to reduce the residual solvent. Cell viability was slightly higher than 100.0% for the S-CNF, presumably for the reason that water-soluble collagen may promote cell proliferation.[5]

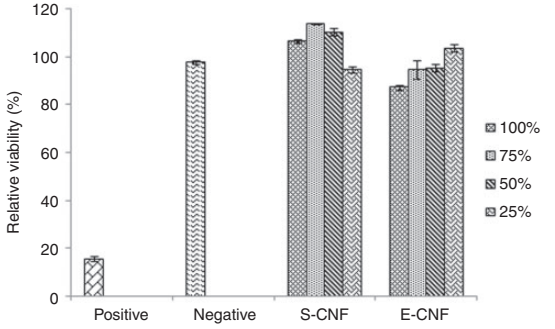

Figure 5. The cytotoxicity test results of S-CNF and E-CNF.
S-CNF: self-assembled collagen fiber; E-CNF: electrospun collagen fiber.

The mean hemolytic rate was 0.31% and 2.87% for S-CNF and E-CNF, respectively, which were both below 5.0%. Therefore, these results indicated that the cytotoxicity and hemocompatibility of S-CNF and E-CNF both met the requirements of medical grade materials.

Skin sensitization test. In the GPMT, few erythema and edema were observed during the experimental periods in the animals from S-CNF, E-CNF and negative control group. The scores of S-CNF and E-CNF were "0" according to criteria proposed by Magnusson and Kligman, which indicated that there was no potential skin sensitization for both S-CNF and E-CNF. Currently, the use of terrestrial animal collagen was more common than that of fish collagen, partly because of the risk concern on the potential sensitization of fish collagen. However, our study found that collagen from tilapia skin had not sensitization. That was why tilapia

had been one of the biggest sources of aquatic edible protein.

Acute systemic toxicity test. The acute systemic toxicity of S-CNF and E-CNF was tested by subcutaneous implantation in BALB/c mice. Animals in all groups grew well with no exercise reduction, diarrhea, ptosis and dyspnea at 4, 24, 48, and 72 hours after implantation. As shown in Figure 6(B), the animal weight in same group increased with time, but there was no significant difference ($p > 0.05$). Moreover, there was also no significant difference in weights between test groups at the same time point. Therefore, the results indicated that the acute systemic toxicity of S-CNF and E-CNF on mice was not observed.

Mouse immunization

The sensitization test was currently used to evaluate the influence of biological material on immune system. However, the sensitization test is not sufficient for the evaluation of immunotoxicity because of the complexity of the immune system. The assessment of immunotoxicity may be performed in five different aspects: inflammation, immunosuppression, immune stimulation, hypersensitivity, and autoimmunity as described in ISO/TS 10993-20:2006. ISO/TS 10993-20:2006 suggests several primary tests to be carried out, such as the weight of immune organs, number of immunocyte and immunoglobulin. Three classical tests including serum IgG titers, weight coefficient of immune organ and lymphocyte proliferation were utilized in our study. As the standard animal for immunization test, BALB/c mice were implanted subcutaneously with collagen samples using a trocar (Table 1). Compared with open surgery, trocar could reduce trauma to animals during implantation.

Antibody analysis. Serum IgG plays an important role in humoral immunity. It was analyzed by ELISA and the result is represented in Figure 6(A). There was no significant change in the titer of IgG between each collagen group and negative control group at 2 weeks and 4 weeks after implantation. The titer of IgG antibody at 8 weeks obviously decreased as compared to that at 2 weeks for all the test groups. The higher titer of IgG at 2 and 4 weeks was conjectured to the trocar operation. However, significant differences were observed between each collagen group and negative control group at

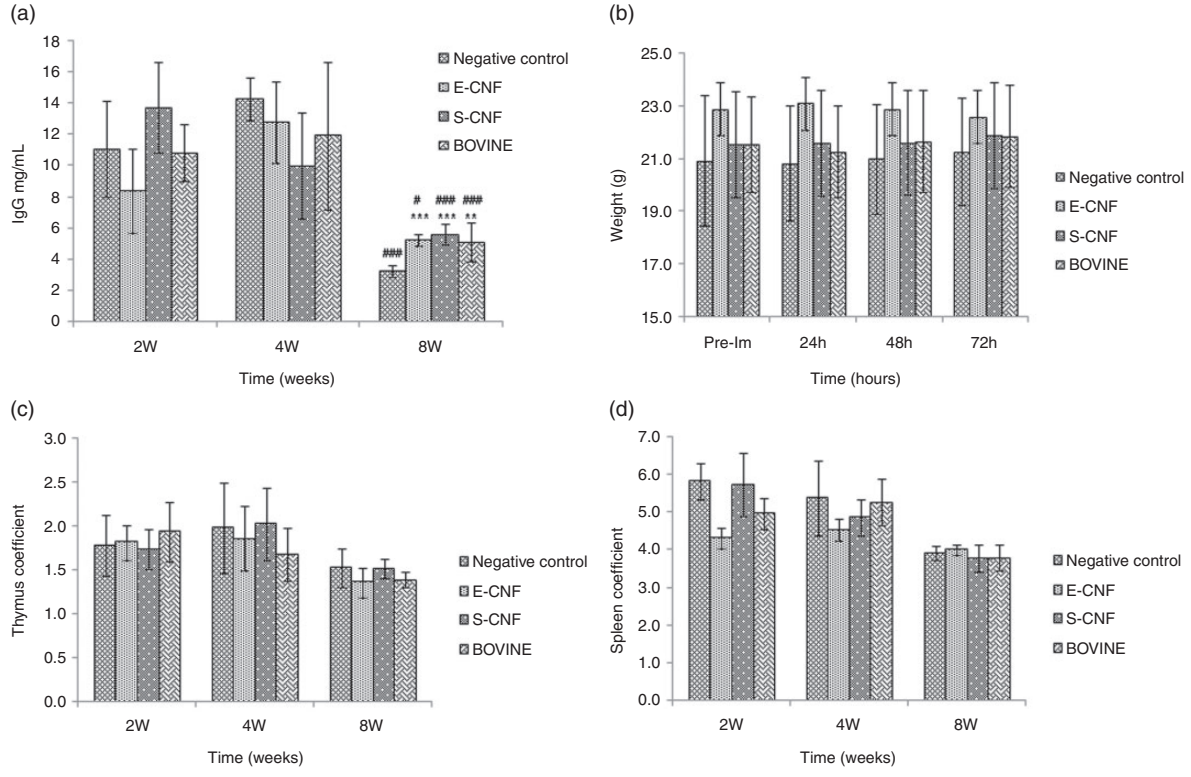

Figure 6. The assessment of the immunological response and acute systemic toxicity of S-CNF and E-CNF after immunization. (A) Serum IgG analyzed by ELISA; (B) the weight of animals before immunization (Pre-Im) and at various time points; (C) thymus coefficient; (D) spleen coefficient (compared to negative control group at the same time point, $^{**}p < 0.01$, $^{***}p < 0.001$; compared to the corresponding group at 2 week, $\#p < 0.05$, $\#\#\#p < 0.001$).
S-CNF: self-assembled collagen fiber; E-CNF: electrospun collagen fiber.

8 weeks ($p < 0.01$). The result showed that S-CNF and E-CNF as well as bovine collagen induced some immunogenicity in mice at the later phase. However, the titer of IgG antibody of S-CNF group and E-CNF group was not significantly different as compared to that of bovine collagen group ($p > 0.05$) at all the time points. This proved that the immunogenicity of S-CNF and E-CNF was equivalent to that of commercial bovine collagen products.

Weight coefficient of immune organs. Thymus coefficient and spleen coefficient were calculated at 2, 4 and 8 weeks after implantation and the results are presented in Figure 6(C,D), respectively. Although there were some differences in the trending of coefficients among experimental groups, the thymus coefficient and spleen coefficient at 8 weeks after implantation both descended to the lowest for all groups. Besides, there was no significant difference ($p > 0.05$) among all the groups for both thymus coefficient and spleen coefficient at 8 weeks, which demonstrated that immune organs all returned to normal level at 8 weeks after implantation for all the groups.

Lymphocyte proliferation test. Lymphocyte proliferation and transformation play an important role in immune response. In this study, lymphocytes were extracted from spleen of BALB/c mice to evaluate the immunogenicity in vitro according to ASTM F1906-98(2003). The result is shown in Figure 7. The Ab of positive group increased significantly as compared to the negative group ($p < 0.01$), which indicated that ConA induced distinct proliferation of lymphocytes. No obvious difference was found between S-CNF group and negative group ($p > 0.05$). However, the Ab of E-CNF group significantly decreased as compared to negative group ($p < 0.01$). It proved that both S-CNF and E-CNF did not induce the cellular immunity effect, yet E-CNF could inhibit lymphocyte proliferation because of its residual hexafluoroisopropanol, in consistence with the result from the cytotoxicity and hemolysis test.

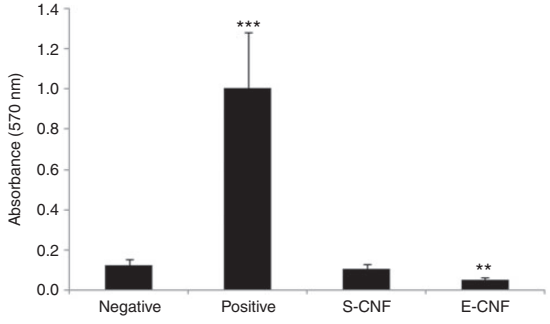

Figure 7. The result of mouse splenic lymphocyte proliferation for S-CNF and E-CNF (compared to negative group, $^{**}p < 0.01$, $^{***}p < 0.001$).
S-CNF: self-assembled collagen fiber; E-CNF: electrospun collagen fiber.

As mentioned above, S-CNF and E-CNF based TSC showed good biocompatibility and low immunogenicity. Thus, TSC could be fabricated into various scaffolds with different density, thickness and pore size as required by self-assembly or electrospinning for tissue-engineering applications.

Conclusion

In this study, two collagen micro/nanofiber materials, S-CNF and E-CNF, were prepared using TSC. Their biocompatibility and immunogenicity were systematically investigated. The result revealed that the state of TSC in S-CNF and E-CNF differs. Collagen in S-CNF retained the triple helical structure of the native collagen, whereas collagen in E-CNF was denatured into gelatin. Nevertheless, all tests for S-CNF and E-CNF including cytotoxicity, hemolysis, skin sensitization and acute systemic toxicity were negative with regardless of whether collagen was in the native or denatured state. Moreover, S-CNF and E-CNF did not induce the proliferation of lymphocytes, and the titer of IgG antibody of S-CNF and E-CNF was comparable to that of commercial bovine collagen. The present study proved that the immunogenicity of S-CNF and E-CNF was also very low. In a separate study, we have observed that S-CNF had good hemostatic function and E-CNF could be used for guided bone regeneration, which will be organized and published later.

In conclusion, the good bio-safety of TSC in its native and denatured state demonstrates that TSC has excellent processability. TSC would be a strongly promising biomaterial for the industry of regenerate medical products and tissue engineering products.

Declaration of Conflicting Interests

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