One-pot Fabrication of Cellulose-collagen Fibrous Networks for Potential Use as Wound Dressing: From Characterization to First Evaluation of Cytocompatibility

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Biomedical researchers have been attempting to construct a wound dressing with the structure and function of a bionic natural extracellular matrix. This dressing would provide a comfortable environment for wound self-healing. In this study, cellulose-collagen fibrous networks were easily fabricated *via* the one-pot method using genipin *in situ* crosslinking collagen hydrolysate in cellulose nanofibrous membranes made by electrospinning cellulose acetate and subsequent deacetylation. The morphology, properties, and successful entrapment of collagen in the cellulose fibrous dressings were validated by scanning electron microscopy, element analysis, Fourier transform infrared spectroscopy, X-ray diffraction, water-swelling test, and thermal gravimetric analysis. The functional cellulose nanofiber-based composite membranes exhibited a network structure, good thermal stability, and acceptable water resistance. Human epidermal cells seeded on the composite nanofibrous membranes presented favorable growth, indicating good cytocompatibility and suitability of the dressing to the wound. Therefore, these novel cellulose-collagen fibrous networks may have potential use in biomedical applications.

Keywords: Cellulose; Collagen; Wound dressing

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INTRODUCTION

Skin injuries caused by abrasions, burns, cuts, falls, violence, and contact with corrosive substances or chronic conditions are inevitable (Gao *et al.* 2019; Gonzaga *et al.* 2019). Wound dressings can provide a physiologically conducive environment that prevents the invasion of bacteria and promotes the self-repair of wounds smoothly to address these problems (Tumolva *et al.* 2019; Koudehi and Zibaseresht 2020). Thus, constructing an effective wound dressing would be greatly beneficial and necessary.

So far, several kinds of wound dressings have been prepared, for example, rubber (Phaechamud *et al*. 2016), foam (dos Santos *et al.* 2018), membrane (Tamer *et al.* 2018), and hydrogel (Mousavi *et al*. 2019; Olivetti *et al*. 2019) *etc*. Researchers have shown considerable interest in a variety of nanotechnologies for the preparation of wound dressings. Electrospinning is a facile and efficient method for producing fibers with a diameter of tens to thousands of nanometers (Han and Steckl 2019). Materials composed of nanofibers are regarded as an outstanding wound dressing matrix due to their high

specific surface area and excellent porosity for the adsorption of wound exudate (Asadi *et al*. 2019). However, these assorted types of wound dressings still have some disadvantages, including cost-ineffectiveness, unnatural sources, and poor biocompatibility. Therefore, developing a wound dressing using a different material is desired to address these drawbacks. Many natural or synthetic polymers, such as cellulose, chitosan, collagen, polyvinyl alcohol, and polycaprolactone, as well as their blends, have been considered for their potential application in wound dressings because of their biocompatibility, biodegradability, low toxicity, low cost, and other excellent properties (Li *et al*. 2017; Poornima and Korrapati 2017; Saeed *et al.* 2017; Hu *et al.* 2018; Olivett *et al.* 2019; Portela *et al*. 2019).

Biopolymer-like proteins have demonstrated superior advantages among the materials that have been used for wound care applications. Collagen and collagen-based materials have been successfully used in medicine over the last 50 years (Shekhter *et al* 2019). Collagen hydrolysate (CH), a polypeptide with a lower molecular weight obtained by hydrolyzing collagen, has recently received attention. Moreover, cellulose, which is derived from plants or microorganisms (Cheng *et al*. 2018; Cai *et al*. 2019), is widely found in nature as a natural polymer. It also has attracted considerable interest from biomedical scientists (Dumanli 2017). As a polysaccharide, it possesses beneficial properties for wound healing due to its biocompatibility, biodegradability, nontoxicity, hydrophilicity, and suitability for chemical modification (Carvalho *et al.* 2019; Portela *et al*. 2019). Therefore, researchers have developed cellulose–collagen composites as biomedicine materials (including wound dressing). Cellulose nanofibers (CNFs) are regarded as a good candidate for wound dressing material owing to their high surface area, chemical and mechanical stability, and ability to mimic the topographical features of the extracellular environment (Gao *et al.* 2019). These properties can offer sufficient space for cell adhesion, migration, and tissue formation. However, few studies have reported on the production of wound dressing using collagen and cellulose-derived nanofibers and their potential application.

In this work, cellulose-collagen membranes were fabricated with a nanofibrous network structure for potential use as wound dressings. Cellulose nanofibrous membranes were fabricated by electrospinning cellulose acetate (CA) and then deacetylation. These membranes were used as a substrate to crosslink collagen via a one-pot facile method. To the best of our knowledge, this study is the first report on the use of fibrous networks constructed by electrospun CNFs and collagen as a novel wound dressing. The physical and chemical characteristics of the composite nanofibrous membranes were examined by scanning electron microscopy (SEM), element analysis, Fourier transform infrared (FTIR) spectroscopy, thermal gravimetric analysis (TGA), X-ray diffraction (XRD), and water-swelling test. Furthermore, the cell-material interactions of the cellulose-collagen fibrous networks were evaluated by human epidermal cell adhesion.

EXPERIMENTAL

Materials

Cellulose acetate (CA, M_n = ~ 300,000) was purchased from Sigma-Aldrich (St. Louis, MO, USA), Collagen hydrolysate (CH) and genipin were obtained from Henan Qiaoshou Food Additives Co., Ltd. (Zhengzhou, China) and Macklin Biological Technology Co., Ltd. (Shanghai, China), respectively. All other chemicals, such as

acetone, sodium hydroxide (NaOH), and N,N-dimethylacetamide (DMAc) were provided by Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All reagents were analytic grade and used directly without further purification. Deionized water was used in the experimental process.

Preparation Procedures

Preparation of cellulose nanofibrous network

The preparation of cellulose nanofibrous network was performed following the methods described by the authors in previous studies with minor modification (Cai *et al.* 2015, 2016, 2017, 2018; Zhang *et al.* 2020). In brief, a homogeneous 16% CA solution (wt%) was obtained by magnetically stirring the mixture of CA and acetone/DMAc (v: v = 2:1) solution for 12 h at room temperature and then loaded into a 10 mL medical syringe. The CA nanofibers (CANFs) were prepared by optimizing the relevant parameters of electrospinning as follows: 20 kV high voltage, 1 mL/h flow rate of the spinning solution, and a 15 cm distance between the needle and the grounded roller receiver with one-layer aluminum foil. Thereafter, the as-obtained CANFs were dried to volatilize the residual solvent at room temperature for 24 h. The cellulose nanofibrous network was fabricated through the deacetylation of CANFs. The CANFs were specifically immersed into a NaOH–ethanol solution (0.1 M) for complete deacetylation for 24 h at room temperature and then washed with deionized water several times. CNFs were obtained by oven drying the above sample at 80 °C for 24 h.

Fabrication of cellulose-collagen nanofibrous networks

The cellulose-collagen composite nanofibrous membrane was synthesized via facile one-pot reaction between collagen and CNFs with genipin as the crosslinking agent. The cellulose nanofibrous membranes of the CNFs were treated with various concentrations of CH aqueous solution (wt%) for 24 h and then immersed into the concentration of 2 mg/mL genipin aqueous solution for 2 h. Furthermore, the cellulose-collagen nanofibrous network was obtained by drying the samples listed above at room temperature. These samples were marked as cro-CNF-CH. The composite membranes uncrosslinked by genipin were correspondingly marked as CNF–CH.

Methods

Characterization

The surface morphology of the as-prepared samples was observed by SEM on a Hitachi S-3000N microscope (Hitachi, Tokyo, Japan). FTIR spectra were investigated using a NEXUS670 spectrometer (Thermo Fisher Scientific, Fitchburg, WI, USA) to analyze the chemical structures with a KBr pellet. The XRD patterns were obtained using a Bruker D8 Advance X-ray diffractometer (PANalytical, Almelo, Netherlands) with Cu-Kα radiation and 2θ from 5° to 80°. TGA was performed on a TGA/DSC/1100SF instrument (Mettler Toledo, Zurich, Switzerland) to evaluate the thermal stability of samples in a N_2 atmosphere with a heating rate of 10 °C/min from 30 °C to 800 °C. The N contents in the samples were measured by a Vario El Cube elemental analyzer (Elementar Analysensysteme GmbH, Langenselbold, Germany).

Swelling tests

The samples were vacuum dried at 60 °C for 24 h before and after immersion in distilled water at room temperature to test the percentage of swelling weight loss as calculated by Eq. 1,

$$\text{Weight loss (\%)} = [(W_d - W_s) / W_d] \times 100 \quad (1)$$

where W_d is the initial weight (g), and W_s is the final weight (g).

Cell Compatibility Study

The biocompatibility of the samples was evaluated by observing the growth of human epidermal cells attached to CNFs, CNF-CH, and cro-CNF-CH. Three samples were immersed in Dulbecco's modified Eagle's medium and then vaccinated with human epidermal cells. Furthermore, the medium was transferred at 37 °C/5% CO_2 atmosphere for 24 h to culture the cells. All operation processes were conducted in a sterile environment. The growth state of the cells dyed on the samples was investigated with laser scanning confocal microscope (Olympus, Tokyo, Japan)

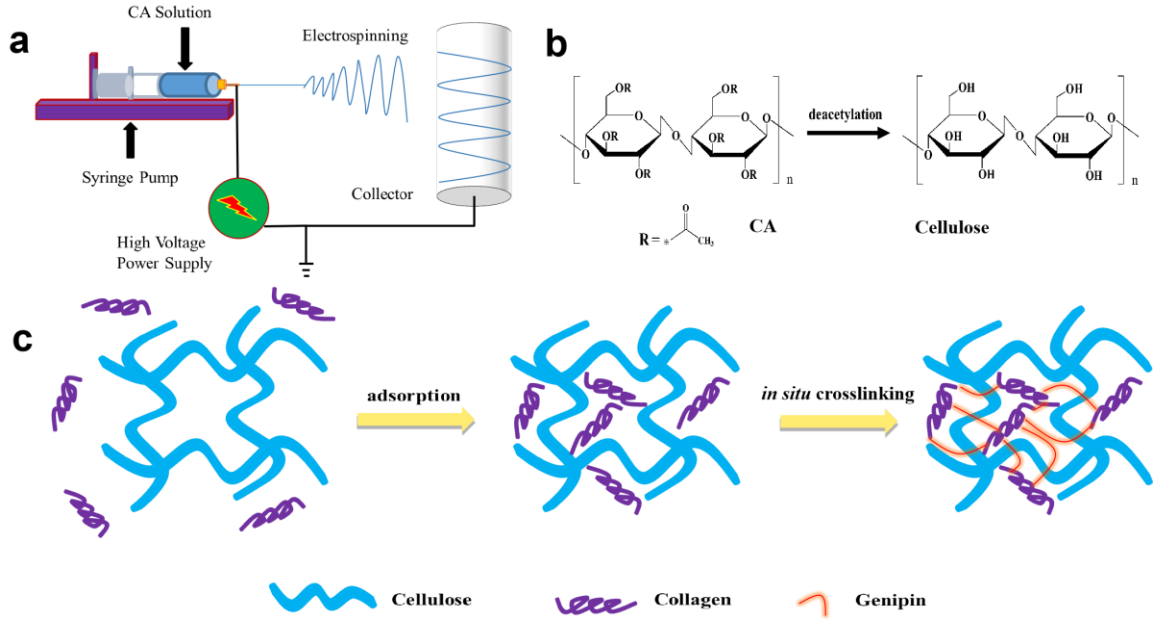

Fig. 1. Schematic diagram of the preparation of cellulose-collagen fibrous networks: (a) preparation of CA nanofibers *via* electrospinning (referring to Zhang *et al*. 2020), (b) structure of CA-to-cellulose, and (c) fabrication of cellulose-collagen nanofibrous membranes by *in situ* crosslinking

RESULTS AND DISCUSSION

Microscopic Images

A schematic of the preparation process of cellulose-collagen nanofibrous networks is shown in Fig. 1. The microscopic appearances of CANFs, CNFs, CNFs-CH, and cro-CNFs-CH are displayed in Figs. 2a, 2b, 2c, and 2d, respectively. Uniform and smooth nanofibers were observed from the SEM images of CANFs and CNFs, indicating that the morphology of nanofibrous structures was maintained after deacetylation. However,

rougher nanofibers (CNFs-CH and cro-CNFs-CH) with thicker diameters (Figs. 2c and 2d) were observed after treatment by CH solution. Genipin was further introduced to crosslink the functional amine groups present in collagen, thereby promoting the formation of network structures. The schematic illustration of CH crosslinked *via* genipin is presented in Fig. 1c. The cro-CNFs-CH (Fig. 2d) showed smoother surfaces than the CNFs-CH (Fig. 2c), because the collagen phase was introduced into cellulose and not easily identified. Moreover, the cellulose composite nanofibrous membranes that were crosslinked with the network system exhibited a compact structure but maintained the original cellulosic skeleton, thereby helping absorb the blood from the wound and preventing the invasion of bacteria in the air. These changes can be explained by the conjecture that the genipin crosslink agents in the protein system may promote the formation of network structures, resulting in the cro-CNFs-CH having both cellulose framework and protein-crosslinked network (Pei *et al.* 2013).

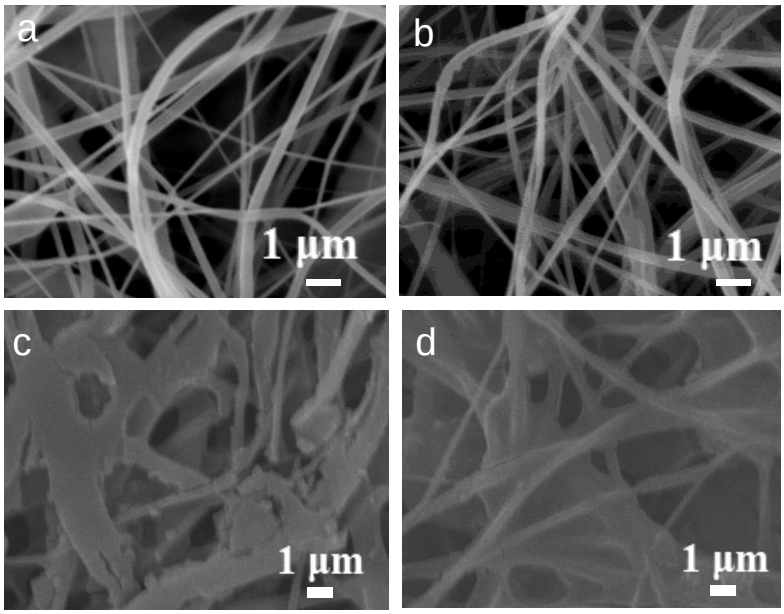

Fig. 2. SEM images of (a) CANFs, (b) CNFs, (c) CNFs-CH, and (d) cro-CNFs-CH

The color of the cro-CNFs-CH (blue) changed compared with that of CNFs (white) (Fig. 3a) due to the production of gardenia blue pigment after the reaction between genipin and the amino acids of collagen. The N content was detected to prove the incorporation of collagen into the composites (Fig. 3b). The N content value of CNFs was 0, which was consistent with the observation that no N element was present in the glucose molecule. Cro-CNF-CH had obvious N content compared with CNFs, suggesting that collagen moved into the CNF matrix.

Information on the relevant chemical bond changes in the samples is shown in Fig. 4a. Relevant analysis was referred from the previous reports (Deng *et al.* 2013; Kuzmenko *et al*. 2014). For the FTIR spectrum of CANFs, the sharp and narrow peak at ~1743 cm^{-1} and ~1369 cm^{-1} were assigned to the C=O stretching vibration of acetyl groups and the -CH_3 deformation vibration, respectively. The sharp and narrow peak at ~1743 and ~1369 cm^{-1} were assigned to the C=O stretching vibration of acetyl groups and the -CH_3 deformation vibration, respectively, for the FTIR spectrum of CANFs. In addition, the strong peaks at ~1238 cm^{-1} and ~1049 cm^{-1} were attributed to the C-O asymmetric bridge

stretching and the skeletal vibration of C-O-C located in the glucopyranose ring, respectively. The strong acetyl carbonyl adsorption peak noticeably disappeared after deacetylation, indicating that CANFs were completely converted to CNFs. In the spectrum of cro-CNFs-CH, the protein (carbonyl group) amide I and amide II bonds were located at ~1643 cm^{-1} and ~1546 cm^{-1}, respectively. The appearance of these two peaks suggested that CH was present on the cellulose matrix, a result that is consistent with that of SEM and element analysis. A similar study confirmed that the absorption bands of CH can be observed in composite nanofibrous membranes prepared without using a crosslinking agent (Pei *et al*. 2013). The XRD patterns of the samples are plotted in Fig. 4b. CANFs showed two broad amorphous peaks, but CNFs exhibited three typical crystalline peaks at 2θ = ~ 12.5°, ~ 20.5°, and ~ 22.4°, which corresponded to the (101), (10$\bar{1}$), and (002) planes of the cellulose crystallines, respectively. Deacetylation was noticeably beneficial to the formation of cellulose crystal structure. The diffraction patterns of cro-CNFs-CH were similar to those of CNFs, that is, the crystallinity was primarily because of cellulose due to the low crystallinity of CH after crosslinking.

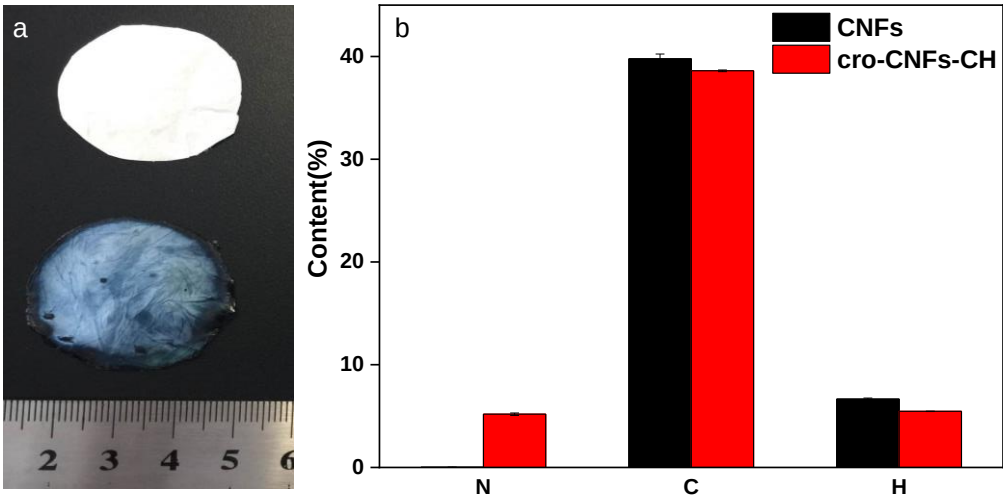

Fig. 3. (a) Typical pictures and (b) elemental analysis chart of CNFs and cro-CNFs-CH

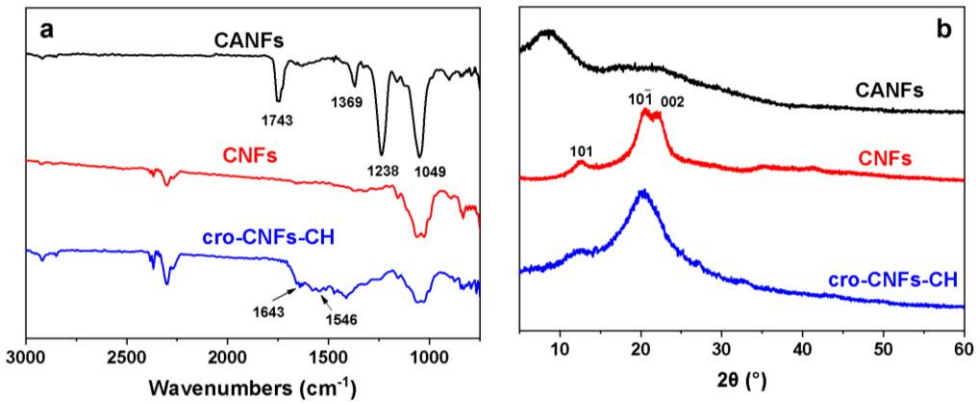

Fig. 4. (a) FTIR spectra and (b) XRD patterns of CANFs, CNFs, and cro-CNFs-CH

The water-swelling behaviors of the various composite nanofibrous membranes are shown in Fig. 5a. The composite nanofibrous membranes generally exhibited less weight loss after crosslinking compared with the membranes with no crosslinking. The weight loss

of both samples increased with increasing concentrations of CH and was caused by the dissolution of CH. The weight loss of the composite membranes after crosslinking noticeably decreased, thereby suggesting the improvement of water resistance of cro-CNFs-CH. The thermal stabilities of the composite nanofibrous membranes were further investigated. The TGA curves for the weight loss of the samples as a function of temperature are plotted in Fig. 5b. The three different steps of decomposition for CNFs are as follows: (1) a slight drop in weight caused by the evaporation of the water of cellulose from 30 ℃ to 200 ℃; (2) a rapid decrease in the mass attributed to the decomposition of cellulose into small molecular glucose, which led to the breaking of glycosidic bonds in the second stage at temperatures ranging from 300 ℃ to 400 ℃; and (3) the decarboxylation and decarbonylation reactions corresponding to the weight loss (400 ℃ to 800 ℃) in the third stage. The TGA curve of cro-CNFs-CH exhibited a similar trend. However, the cro-CNFs-CH network showed a relatively low rate of weight loss, indicating that the thermal stability of cro-CNFs-CH network was enhanced due to the presence of collagen.

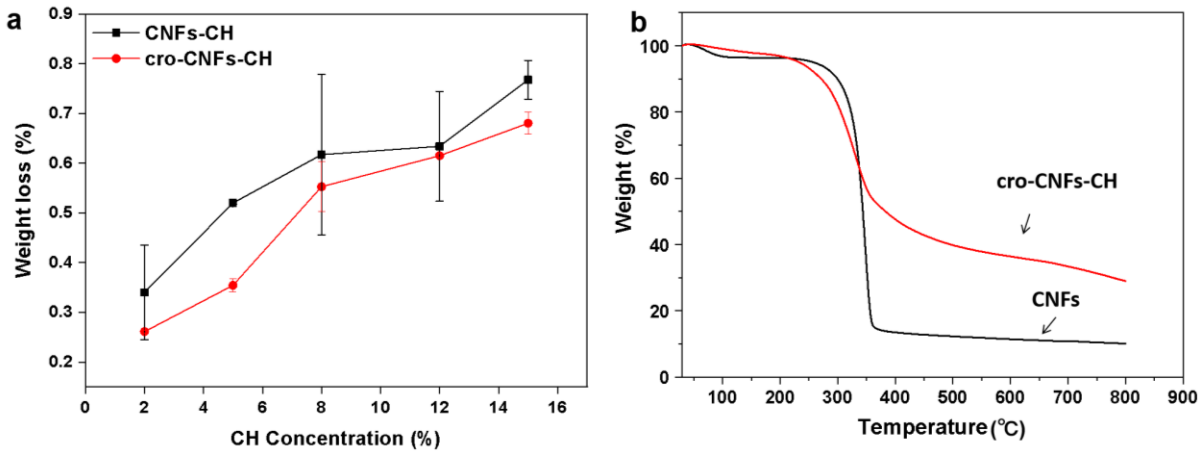

Fig. 5. (a) Water-swelling behaviors of the composite membranes before and after crosslinking and (b) TGA curves of CNFs and cro-CNFs-CH

Wound dressing materials should possess good biocompatibility in the practical applications. Collagen can provide a natural extracellular environment that supplies nutrition for the cell, which is important for cell communication and layer formation. The biocompatibility of composite membranes must be evaluated *via* evaluated by the corresponding cell compatibility assessments. Herein, human epithelial cells were seeded on the surfaces of CNFs-CH and cro-CNFs-CH to determine the cellular proliferation. CNFs were used as a control. Results of the human epidermal cells culture based on the different samples are shown in Fig. 6. After a 24 h culture period, the cells proliferated normally on the CNFs (Fig. 6a), CNFs-CH (Fig. 6b), and cro-CNFs-CH (Fig. 6c) matrixes, preliminarily suggesting that the as-prepared cro-CNFs-CH membrane possessed good biocompatibility. The present study demonstrated the advantages and potential of the cro-CNFs-CH and provides a basis for further study of its practical application as a wound dressing in the biomedical field.

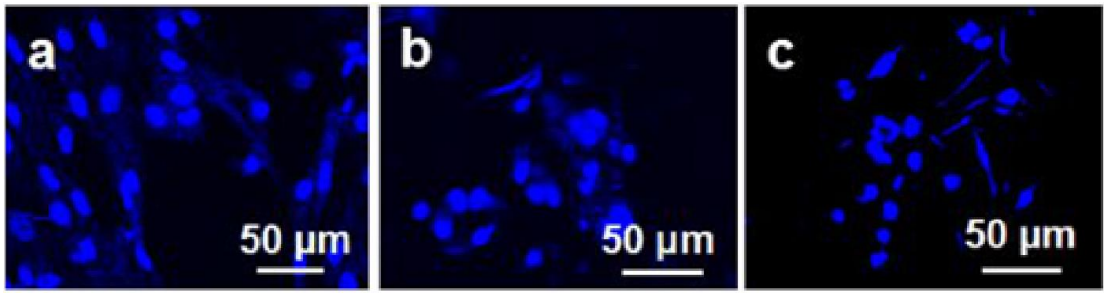

Fig. 6. Human epidermal cells cultured on various samples: (a) CNFs, (b) CNFs-CH, and (c) cro-CNFs-CH

CONCLUSIONS

Cellulose-collagen nanofibrous network membranes were robustly synthesized via the one-pot sample method. The properties of the composite nanofibrous membranes were characterized by several techniques, including SEM, element analysis, FTIR, and XRD, which were used to confirm the successful preparation of cellulose-collagen membranes with a nanofibrous network structure.

1. The cellulose-collagen nanofibrous membranes cross-linked through genipin further improved their water resistance properties in distilled water and their thermal stability was enhanced.
2. Results of the cell culture study proved that the cellulose-collagen nanofibrous membranes could support cell adhesion and proliferation, thereby suggesting good biocompatibility.
3. A facile route for creating the cellulose-collagen fibrous network would serve as a practical application in wound dressing.

ACKNOWLEDGMENTS

Dr. Jie Cai thanks the Young Elite Scientists Sponsorship Program by CAST (No. 2018QNRC001) and the Chutian Scholar Program of Hubei Provincial Government, China. This work was also partially funded by the Hubei Key Laboratory for Processing and Transformation of Agricultural Products (WHPU, China) (No. 2018HBSQGDKFB01), the Talent Introduction Foundation of WHPU (No. 2016RZ22), the Research and Innovation Initiatives of WHPU (No. 2018J01), and the Province Key Laboratory of Cereal Resource Transformation and Utilization, Henan University of Technology (No. PL2018001).

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Article submitted: October 14, 2019; Peer review completed: January 14, 2020; Revised version received and accepted: February 12, 2020; Published: February 20, 2020.
DOI: 10.15376/biores.15.2.2501-2511