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Short communication

Lyophilization as a novel approach for preparation of water resistant HA fiber membranes by crosslinked with EDC

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ABSTRACT

The hyaluronic acid (HA) fibers scaffold with an extracellular matrix mimic structure has been prepared via lyophilization. The morphology of the HA fibers varying the concentration from 0.05 wt% to 0.15 wt% was characterized by scanning electron microscope (SEM). The diameter of HA fibers increased and the morphology changed from fiber to sheet-like structure as the concentration of HA solution increased. To enhance the water-resistance, the pure HA fiber membranes were chemical cross-linked by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), and confirmed by fourier transform infrared (FT-IR) spectra. In vitro degradation behavior of cross-linked HA fiber membranes in both of water and PBS solutions was investigated, the physical properties were also studied by differential scanning calorimetry (DSC) and thermogravimetry (TG). The results showed that the bonding water capacity increased after crosslinking, and indicated that the crosslinked HA fibers could be used as scaffold in tissue engineering.

1. Introduction

Tissue engineering using three-dimensional (3D) porous scaffolds provided support for the tissue regeneration (Collins & Birkinshaw, 2013). The 3D substrate serves as a template for tissue regeneration. The ideal scaffolds should have an appropriate surface chemistry and microstructures to facilitate cellular attachment, proliferation and differentiation (Ma, Kotaki, Inai, & Ramakrishna, 2005). The fabrication of the applicable scaffolds was the core task of tissue engineering. The high surface area and interconnected 3D pore system were considered as the key factor for scaffolds due to facilitating cell attachment and permitting the diffusion of nutrients. Thus, various techniques, including solvent casting/salt leaching, phase separation, emulsion freezing/drying (Kim, Chung, & Park, 2008; Lam et al., 1994; Naznin & Wang, 2012; Pakavadee & Masahiro, 2012) have been developed for the preparation of foam-like scaffolds with the highly porous structure. Recently, the fabrication of bio-mimic architecture to mimic the native extracellular matrix (ECM), which provided an intricate fibers web for cell adhesion, has been driven an increasing interest.

The fibers were suitable for tissue engineering scaffold, due to the continuous structure, high porosity, high surface-to-volume ratio and natural ECM mimic morphological which is the most important characteristic (Li et al., 2006a; Li, He, Han, & Zheng, 2006b). Various strategies, such as self-assembly (Zhang & Eisenberg, 1998) and electrospinninng (Ji et al., 2006), have been developed to mimic the architecture of the natural ECM. Electro spinning fibers have been extensively applied in the tissue engineering because of their closely mimic of the EMC architecture (Rajesh & Dhirendra, 2006). However, some limitations of electrospinnability in natural macromolecules, such as gelatin, chitosan and hyaluronic acid (Li et al., 2006a; Li et al., 2006b; Ohkawa, Cha, Kim, Nishida, & Yamamoto, 2004), restricted its application in natural materials fibers.

In order to improve the water-resistance properties, hyaluronic acid (HA), found ubiquitously in the ECM of virtually all mammalian connective tissues (Collins & Birkinshaw, 2008; Laurent, Laurent, & Fraser, 1995), has been served as a potential tissue engineering scaffold material cross-linked with DVS, epoxide, formaldehyde, glutaraldehyde or genipin because of the excellent water-hold properties to mimic the microenvironment of cell regeneration (Burns, Burgess, & Skinner, 1996; Eun et al., 2008; Tan, Li, Rubin, & Marra, 2011; Tomihata & Ikada, 1997; Zhao, 2000).

Lyophilization is a simple and versatile route for the preparation of 3D monolithic structures using ice as the template (Collins & Birkinshaw, 2011; Qian & Zhang, 2011). An aqueous solution is frozen following with sublimation of the ice to template the 3D architecture of ice crystals. It is a flexible technology that can be applied to a range of materials such as natural macromolecule,

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polyelectrolyte and polymer colloids to prepare the 3D scaffold. Versatile complex ordered structures have been fabricated via lyophilization by controlling the freezing parameters. However, few articles have been reported to fabricate fiber membranes via lyophilization.

Herein, HA fibers were prepared by the lyophilization with the HA solutions and subsequently cross-linked with EDC, and then the water-resistance property was enhanced for the application of tissue engineering. The influence of the concentration ranging from 0.05 wt% to 0.15 wt% on the morphology of the HA fibers has been investigated. Both in water and PBS solutions, in vitro degradation behavior of cross-linked HA fiber membranes were also investigated. It was expected that the cross-linked fiber membranes might have potential applications in tissue engineering.

2. Experimental

2.1. Materials

Hyaluronic acid (HA, M_W = 2,000,000) was purchased from Freda Biopharm. Co., Ltd. (Shandong, China).1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was purchased from Aladdin (Shanghai, China). Ethanol (AR) was purchased from Richjoint Chem. Co., Ltd. (Shanghai, China). The deionized water was prepared by UPT ultrapure water polishing system.

2.2. Preparation of HA fiber membranes

0.05 wt%, 0.15 wt% and 0.3 wt% HA solutions were prepared by dissolving HA in deionized water by gentle agitation in a 250 mL container at room temperature. These solutions were frozen quickly by the liquid nitrogen and freeze-dried by freeze-drier (LGJ-10, Beijing) for 48 h with the temperature about −84 °C and vacuum degree below 1 Pa.

2.3. Preparation of cross-linked HA fiber membranes

The HA fiber membranes were weighed (around 10 mg) and immersed into EDC (50 mM) at 4 °C and kept for 24 h. The cross-linked membranes were washed with deionized water and ethanol respectively for three times, and then dried in vacuum at room temperature for 72 h to remove the residual solvents.

2.4. Characterization

The morphology of the freeze-drying HA fibers was characterized by SEM (S-4700, Hitachi). Each sample was sputter-coated with gold for analysis. FT-IR spectra of the freeze-drying fibers were measured by using a FTIR spectrometer (Nicolet iS5, Thermo Fisher).

To investigate the degradation behavior of the cross-linked HA fibers in vitro, the fibers membranes (about 10 mg) were immersed in 5 mL PBS (pH = 7.4) and deionized water at 37 °C for different time intervals. The degradation rate was evaluated through the weight changes before and after immersion according to Eqn. (1) (Xu et al., 2009).

$$\text{Weight remaining}(\%) = \left(\frac{W_{\text{after}}}{W_{\text{before}}}\right) \times 100 \quad (1)$$

where W_{after} is the remained weight of membranes after immersion in PBS and W_{before} is the original weight. Three samples were averaged as each measurement result. Thermal behavior of HA fibers were investigated by a thermo gravimetric analyzer (TGA, TA Instruments, USA, SDT Q600) with a heating rate of 10 °C/min from 50 °C to 450 °C. DSC (Perkin Elmer Pyris 1) was heated from 50 °C to 250 °C at a heating rate of 10 °C/min.

3. Results and discussion

The fiber membranes were prepared by freezing HA solutions at different concentrations 0.05 wt%, 0.15 wt%, and 0.3 wt%, then cross-linked with EDC for 24 h. The SEM images of HA fibers were shown in Fig. 1. The HA solution frozen in liquid nitrogen with 0.15 wt% concentration showed uniform fiber structure (Fig. 1b),

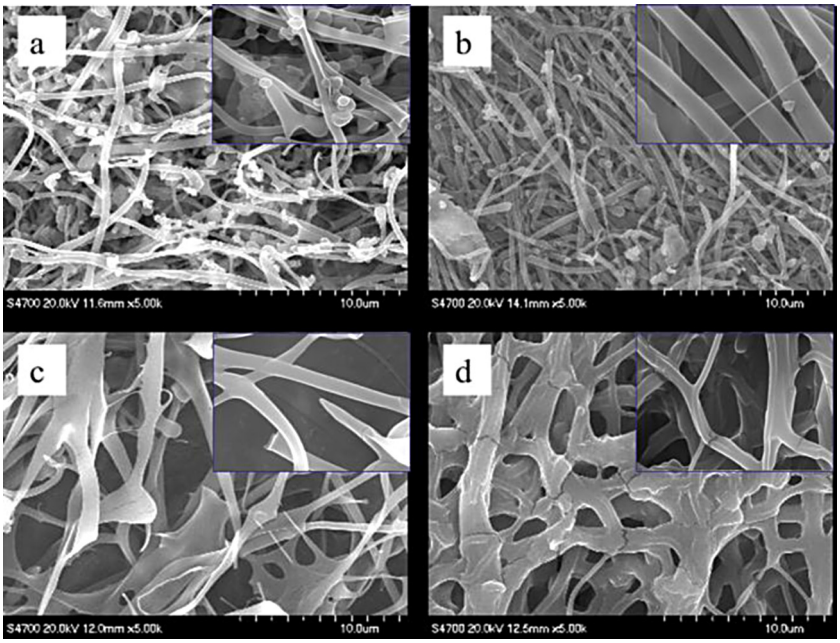

Fig. 1. SEM image of HA fiber membranes lyophilizing at different concentrations, (a) 0.05%; (b) 0.15%; (c) 0.3%, and (d) SEM image of HA lyophilizing fiber membranes cross-linked by EDC.

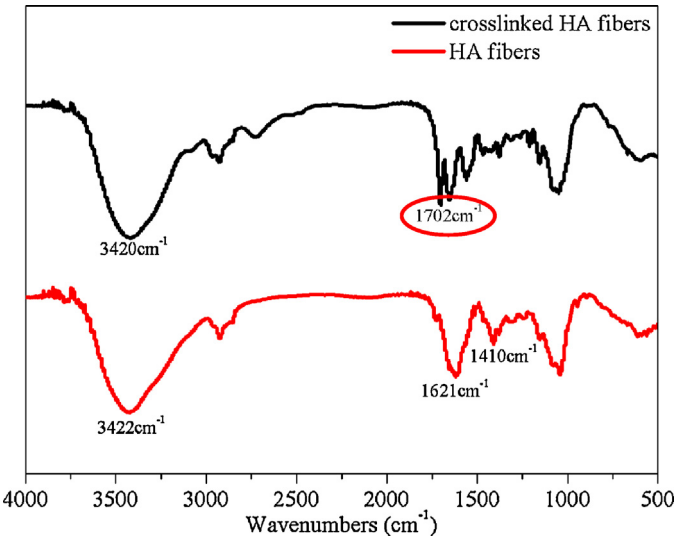

Fig. 2. FT-IR spectra of the pristine and cross linked HA fiber membranes.

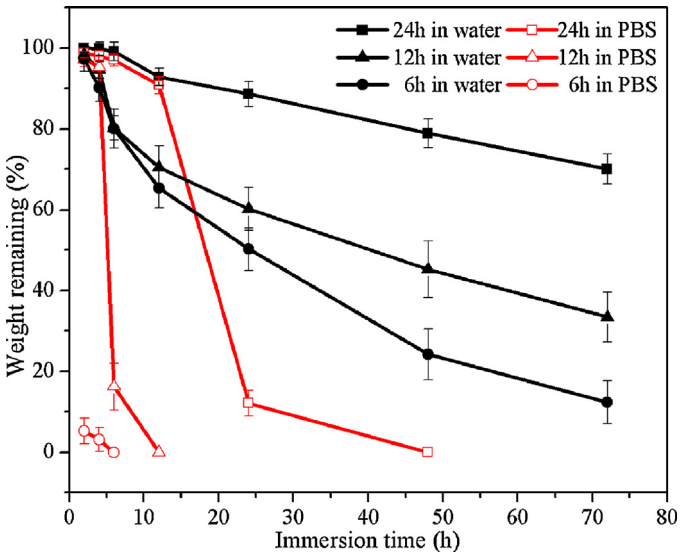

Fig. 3. Degradation behaviors of cross-linked HA fiber membranes in PBS and water at 37 °C.

most of which had ribbon-like structure. The morphology of the fibers changed according to the concentration of the solutions. At the concentration of 0.05 wt%, it showed fiber and granule structure membranes (Fig. 1a). As the concentration increased to 0.3 wt%, it formed sheet-like structure membranes (Fig. 1c). The driven force of the fiber formation was considered as the assembling of the HA molecules between ice crystal interstices. When the HA solution was frozen, ice crystals grew and HA molecules were excluded by these crystals. Due to the poor solubility of HA in ice crystals, the HA molecules dispersed on the surface of the crystals and held together by Van der Waals forces and hydrogen bonds, fibers formed (Butler, 2002). The formation of the fiber-granule structure at low concentration was considered as the lack of HA molecules dispersed in the crystals interstice to form continuous fiber structure. The formation of sheet-like structure might be caused by the colliquefaction of the HA fibers on the surface of ice crystals due to the high concentrations.

The SEM images of the 0.15 wt% fibers crosslinked by EDC were shown in Fig. 1d. It indicated that the fibers morphology of HA membranes was still maintained, but part adhesion and swelling of the fibers was observed after 24 h cross-linked in EDC/Ethanol solution. The diameter of fibers increased forming net-like structures. This might be the result of a long time soak in ethanol solution.

The chemical cross-linking structures of the HA fibers were characterized by FTIR spectra (Fig. 2). The peak at 1621 cm^{-1} was assigned as the characteristic —COOH peak related to disaccharide units of HA, the peak at 1410 cm^{-1} assigned to the carbonyl symmetric absorption peak. Compared with the pristine HA fibers, the peaks at 1621 cm^{-1} and 1410 cm^{-1} were weakened after cross-linked. The noticeable new peak was appears at the wave number of 1702 cm^{-1}, which was assigned to the stretching vibration of carbonyl group for the newly formed ester bond. These results indicated that the —COOH groups took part in the cross-linking reaction initiated by EDC (Xu et al., 2009).

The in vitro degradation behaviors of cross-linked HA fiber membranes were investigated by immersing the fibers into water and PBS respectively at 37 °C, and the results were shown in Fig. 3. The water resistant property of the crosslinked fibers was enhanced as the increase of the cross-linking time. For the 6 h cross-linked fibers, it almost 100% degraded in the first 8 h immersing in the PBS solutions, and only 11.9% remained after 72 h degradation in the water. By prolonging the cross-linking time to 12 h, the water resistant of fibers was slightly improved both in the water and PBS solutions. However, the water resistant property of the fibers after 24 cross-linking was obviously increased that it about 89% remained after 48 h degradation in the water, and 71% at 72 h, but unfortunately it almost 100% degraded of the 24 h cross-linked fibers by immersing in the PBS solutions 48 h. It showed that the water resistant was increased as the increase of cross-linking time,

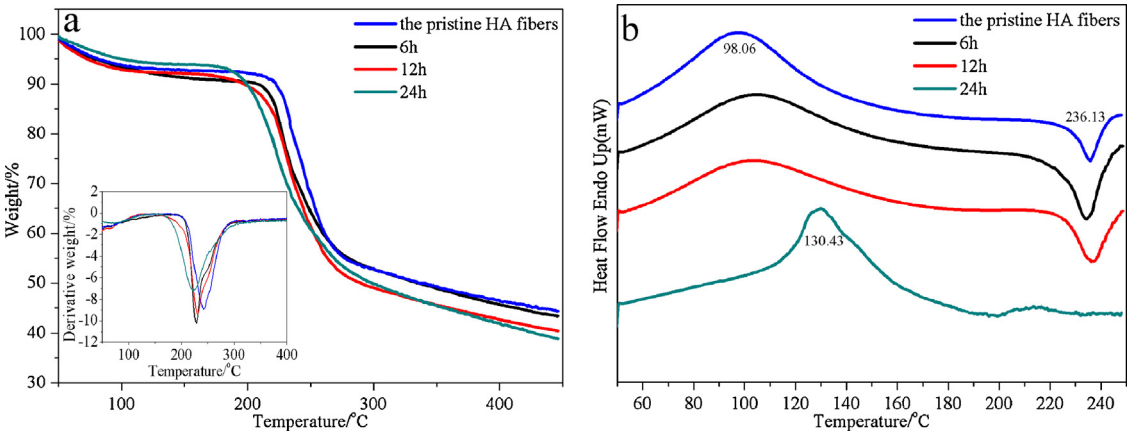

Fig. 4. (a) TGA/DTG and (b) DSC thermo grams of the pristine and cross-linked HA fiber membranes with different cross linking time.

due to the increase of the crosslinking density of HA fibers by prolonging the cross-linking time. It also showed that the cross-linked fiber membranes exhibited a different degradation profile in water and PBS, which might because of the strong ionic environments in PBS caused the ester bond cross-linking the HA molecules more inclining to hydrolyze comparing in the water (Xu et al., 2009).

Thermal analysis of HA fibers was shown in Fig. 4. The TGA analysis (Fig. 4a) showed that the water evaporation reduced as the cross liking time prolonging. The initial thermal decomposition temperature of the pristine HA fibers and the cross-linked fibers for 24 h was 207 °C and 185 °C, respectively, and the residual rate was 36 wt% and 44 wt%, accordingly. For the DSC curves (Fig. 4b), the peak at 98 °C and 130 °C could be attribute to dehydrate water from the pristine HA fibers and the cross-linked HA fibers for 24 h respectively. It could be seen that the dehydration temperature (T_d) of the cross-linked HA fibers for 24 h was higher than that of the pristine HA fibers, and the dehydration enthalpy (ΔH_d) of the pristine HA was 473 J/g, slightly higher than the cross-linked of 463 J/g. For the DSC curves of pristine HA fibers, the peak of HA crystal melt was appeared at 236 °C, but no peak for the cross-linked fibers for 24 h, which indicated that the HA crystal has been destroyed during the cross-linking process.

The decreased of weight loss of cross-linked HA fibers below 180 °C from the TGA result, and the decrease of ΔH_d from the DSC result proved that the water content in the cross-linked HA fibers was less than that in pristine HA fibers. However, the increase of T_d from the DSC analysis indicated that the bonding effects of intermolecular interaction with water have been enhanced after cross-linked (Xu et al., 2009). The decreased decomposition temperature of HA fibers after cross-linked for 24 h was shift from 207 °C to 185 °C, which might caused by the loss of EDC from the cross-linked HA fibers. It indicated that the ester bond of the cross-linked HA was broken before HA decomposition.

4. Conclusion

In this study, HA fiber membranes were prepared by the lyophilization technique. The cross-linked HA fiber membranes by EDC showed improvement in the stability in PBS and water. SEM showed that the diameter of the fibers increased with the increase of concentration of HA solutions. FT-IR results confirmed the formation of ester bond and indicated that the cross-linking reactions occurred and the cross-linked fiber membranes were more stable in PBS and water. DSC and TGA results confirmed that the bonding effect of intermolecular interaction with water has been enhanced after cross-linking.

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References

Burns, J. W., Burgess, L., & Skinner, K. (1996). A hyaluronate based gel for the prevention of postsurgical adhesions: evaluation in two animal species. *Fertility and Sterility*, *66*, 814–821.

Butler, M. F. (2002). Freeze concentration of solutes at the ice/solution interface studied by optical interferometry. *Crystal Growth & Design*, *2*, 541–548.

Collins, M. N., & Birkinshaw, C. (2008). Investigation of the swelling behavior of crosslinked hyaluronic acid films and hydrogels produced using homogeneous reactions. *Journal of Applied Polymer Science*, *109*, 923–931.

Collins, M. N., & Birkinshaw, C. (2011). Morphology of crosslinked hyaluronic acid porous hydrogels. *Journal of Applied Polymer Science*, *120*, 1040–1049.

Collins, M. N., & Birkinshaw, C. (2013). Hyaluronic acid based scaffolds for tissue engineering – a review. *Carbohydrate polymers*, *92*, 1262–1279.

Eun, J., Kang, S., Kim, B., Jiang, G., Il, H., & Sei, K. (2008). Control of the molecular degradation of hyaluronic acid hydrogels for tissue augmentation. *Journal of Biomedical Materials Research. Part A*, *86*(3), 685–693.

Ji, Y., Ghosh, K., Shu, X. Z., Li, B. Q., Sokolov, J. C., Prestwich, G. D., et al. (2006). Electrospun three-dimensional hyaluronic acid nanofibrous scaffolds. *Biomaterials*, *27*, 3782–3792.

Kim, T. G., Chung, H. J., & Park, T. G. (2008). Macroporous and nanofibrous hyaluronic acid/collagen hybrid scaffold fabricated by concurrent electrospinning and deposition/leaching of salt particles. *Acta Biomaterialia*, *4*, 1611–1619.

Lam, K. H., Nieuwenhuis, P., Molenaar, I., Esselbrugge, H., Feijen, J., Dijkstra, P. J., et al. (1994). Biodegradation of porous versus nonporous poly (L-lactic acid) films. *Journal of Materials Science – Materials in Medicine*, *5*, 181–189.

Laurent, T. C., Laurent, U. G., & Fraser, J. E. (1995). Functions of hyaluronan. *Annals of the Rheumatic Diseases*, *54*, 429–432.

Li, J. X., He, A. H., Han, C. C., Fang, D. F., Hsiao, B. S., & Chu, B. (2006). Electro spinning of hyaluronic acid (HA) and HA/gelatin blends. *Macromolecular Rapid Communications*, *27*, 114–120.

Li, J. X., He, A. H., Han, C. C., & Zheng, J. F. (2006). Gelatin and gelatin-hyaluronic acid nanofibrous membranes produced by electrospinning of their aqueous solutions. *Biomacromolecules*, *7*, 2243–2247.

Ma, Z. W., Kotaki, M., Inai, R., & Ramakrishna, S. (2005). Potential of nanofiber matrix as tissue engineering scaffolds. *Tissue Engineering*, *11*, 101–109.

Naznin, S., & Wang, M. (2012). PHBV/PLLA-based composite scaffolds fabricated using an emulsion freezing/freeze-drying technique for bone tissue engineering: surface modification and in vitro biological evaluation. *Biofabrication*, *4*, 015003.

Ohkawa, K., Cha, D., Kim, H., Nishida, A., & Yamamoto, H. (2004). Electrospinning of chitosan. *Macromolecular Rapid Communications*, *25*, 1600–1605.

Pakavadee, R., & Masahiro, O. (2012). Preparation of highly porous β-chitin structure through nonsolvent – solvent exchange-induced phase separation and supercritical CO_2 drying. *The Journal of Supercritical Fluids*, *68*, 31–38.

Qian, L., & Zhang, H. F. (2011). Controlled freezing and freeze drying: a versatile route for porous and micro-/nano-structured materials. *Journal of Chemical Technology and Biotechnology*, *86*, 172–184.

Rajesh, V., & Dhirendra, S. K. (2006). Nanofibers and their applications in tissue engineering. *International Journal of Nanomedicine*, *1*, 15–30.

Tan, H., Li, H., Rubin, J. P., & Marra, K. G. (2011). Controlled gelation and degradation rates of injectable hyaluronic acid-based hydrogels through a double crosslinking strategy. *Journal of Tissue Engineering and Regenerative Medicine*, *5*, 790–797.

Tomihata, K. J., & Ikada, Y. S. T. (1997). Crosslinking of hyaluronic acid with glutaraldehyde. *Journal of Polymer Science Part A: Polymer Chemistry*, *35*, 3553–3559.

Xu, S. S., Li, J. X., He, A. H., Liu, W. W., Jiang, X. Y., Zheng, J. F., et al. (2009). Chemical cross linking and biophysical properties of electrospun hyaluronic acid based ultra-thin fibrous membranes. *Polymer*, *50*, 3762–3769.

Zhang, L. F., & Eisenberg, A. (1998). Formation of crew-cut aggregates of various morphologies from amphiphilic block copolymers in solution. *Polymers for Advanced Technologies*, *9*, 677–699.

Zhao, X.(2000). Process for the production of multiple cross-linked hyaluronic acid derivatives. WO/2000/046253.