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# Hyaluronic acid/EDC/NHS-crosslinked green electrospun silk fibroin nanofibrous scaffolds for tissue engineering

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Regenerated silk fibroin (SF) from *Bombyx mori* was used widely in biomedical fields due to its excellent properties. In recent years, green electrospun SF nanofibers have attracted much attention from researchers due to their good biosafety and environmentally friendly nature. However, the mechanical properties of SF nanofibers are unsatisfactory, which greatly restricts the application of SF in tissue engineering. In this study, the tensile performance of the green electrospun SF nanofibers was significantly improved through a simple and eco-friendly process of hyaluronic acid (HA)/1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)/*N*-hydroxysuccinimide (NHS)-crosslinking. Our data showed that the strain performance of the HA/EDC/NHS-crosslinked SF nanofibrous matrices was dramatically improved up to 120%, and was much better than that of the conventionally treated ones (ethanol soaking or fumigating). The appropriate HA/EDC/NHS-crosslinking time for SF nanofibrous matrices was 24 hours. The strain performance of the as-crosslinked SF nanofibers increased with an increase in HA concentration, with the optimal HA concentration being 0.3% (w/v). Fourier transform infrared spectroscopy analysis suggested that the HA/EDC/NHS-crosslinking process involves covalent reactions. The hydrophilicity of the SF nanofibrous matrices also increased with the addition of HA, which can be useful when trying to resist non-specific protein adsorption. In addition, the as-crosslinked SF nanofibrous matrices exhibited good cytocompatibility as shown by the cell viability data. Our work demonstrated that HA/EDC/NHS-crosslinking is a good choice for improving the tensile properties of the green electrospun SF nanofibers serving as skin tissue engineering scaffolds.

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## Introduction

Regenerated silk fibroin (SF) from *Bombyx mori* is considered to be one of the most promising candidates for tissue engineering applications due to its excellent biocompatibility, biofunctionality, low inflammatory response, and so on.[1–4] In the past decades, SF was widely studied, being processed into films,[5] hydrogels,[6] micro-particles and nano-particles[7] using phase separation, ultrasound dispersion, lyophilization and electrospinning.[6,8–10] The electrospun SF nanofibrous scaffolds have shown impressive advantages for tissue engineering applications, which are commonly attributed to their remarkable capacity to mimic the micro-environment of natural extracellular matrix (ECM).[4,11–18] Recently, a green electrospinning process has been developed to produce SF nanofibers while avoiding harm to the environment and potential toxicity towards cells, during which the SF was dissolved in water,[4,12,19,20] and polyethylene oxide (PEO) was usually added to increase the viscosity of the solution.[13,20,21]

However, it is known that the mechanical properties of the electrospun SF nanofibers are not satisfactory, which greatly limits their application. Commonly used post-spin treatments, which only raise the water-resistance of the SF nanofibers, include methanol[22,23] or ethanol soaking[24] and water[25] or ethanol vapour.[4,12,13,19] In previous studies, we developed a method using 75% ethanol vapour, which skillfully integrated the post-spin treatment with a sterilization process.[13,19] However, all of these post-spin treatment approaches failed to improve the tensile properties of the SF nanofibers. Hence, it is still very urgent to find effective ways to improve the tensile properties of the green electrospun SF nanofibers to meet the requirements of tissue engineering.

Recently, SF/hyaluronic acid (HA) blend materials have emerged due to their excellent properties.[26–28] The SF/HA film had a better mechanical performance than the pure SF film.[27,28] Furthermore, the introduction of HA has the potential to induce

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an SF structural transformation from silk I to silk II.[27] Due to its carboxyl group, HA can be activated and modified by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)/*N*-hydroxysuccinimide (NHS) which was commonly used as a carboxylic acid activation reagent in amide synthesis and is able to introduce some useful groups to the SF through the formation of covalent bonds to primary amines.[29–34] Therefore, we hypothesized that the mechanical properties of the electrospun SF nanofibers might be significantly improved by crosslinking them with both EDC/NHS and HA. In this work, the SF nanofibrous matrices, fabricated by aqueous solution electrospinning, were crosslinked by HA/EDC/NHS and then characterized using a Scanning Electron Microscope (SEM), a universal testing machine, Fourier transform infrared spectroscopy (FTIR) and water contact angles. Moreover, the cytocompatibility of the as-crosslinked materials was evaluated by 3-[4,5-dimethyl thiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), SEM and fluorescence staining assays.

# Experimental

## Materials

Cocoons of *B. mori* silkworm were purchased from Jiaxing Silk Co. (China). PEO ($M_w$ = 900 000), EDC, NHS, 2,4,6-trinitrobenzensulfonic acid (TNBS) and HA were all purchased from Sigma-Aldrich China Inc. L929 cells were provided by the Institute of Biochemistry and Cell Biology (Chinese Academy of Sciences, China). Other chemicals were all of analytical grade or higher and used as received. Ultrapure water was used throughout the whole study.

## Preparation of regenerated SF

*B. mori* silk cocoons were boiled in a 0.5% (w/v) $Na_2CO_3$ aqueous solution for 30 min, three times and then rinsed six times in distilled water to remove the sericin proteins. The degummed silk was left at 40 °C until it was dry and then was dissolved in a ternary solvent system of $CaCl_2/H_2O/CH_3CH_2OH$ solution (1 : 8 : 2 in molar ratio) at 65 °C for 2 h. The solution was dialyzed in ultrapure water with a dialysis bag (MWCO 14 kDa, Sigma-Aldrich) at room temperature for 3 days, with the water being changed four times each day. Finally, the SF sponges were obtained after filtering and lyophilizing the silk solution.

## Preparation of SF nanofibrous matrices

The SF was dissolved in ultrapure water with a final concentration of 20% (w/v) and PEO was added to the SF solution with a final concentration of 2% (w/v). The blend solution was well stirred to be uniform and stable. The solution was then electrospun with a constant feed rate of 0.8 ml $h^{-1}$ under a positive voltage of 10 kV and the distance between the syringe needle and acceptor was 15 cm. In order to remove any residual water vapour, the SF nanofibrous mats were stored and dried under vacuum at room temperature prior to use.

## Crosslinking of the nanofibrous matrices

EDC/NHS was dissolved in 95% ethanol and stirred for 30 min to get a homogeneous solution of EDC/NHS (100 mM/100 mM) solution. Then, different amounts of HA (0.05 g, 0.1 g, 0.3 g and 0.5 g) were dissolved in 1 ml of deionized water to obtain HA aqueous solutions with concentrations of 0.5%, 1.0%, 3.0% and 5.0% (w/v) respectively. 10 ml of each of the HA aqueous solutions was then added to 90 ml of the prepared EDC/NHS solution to form the HA/EDC/NHS crosslinking solution. The final concentrations of HA in the crosslinking solutions were 0.05%, 0.1%, 0.3% and 0.5% (w/v) respectively, and the solutions were then named as HA (0.05%)/EDC/NHS, HA (0.1%)/EDC/NHS, HA (0.3%)/EDC/NHS and HA (0.5%)/EDC/NHS correspondingly.

The nanofibrous matrices were cut into 50 mm × 10 mm portions and crosslinked using a different approach. To compare the crosslinking effect between HA/EDC/NHS and some conventional methods, the samples were treated with 50 ml HA (0.05%)/ethanol, EDC/NHS, and HA (0.05%)/EDC/NHS respectively at room temperature for 24 h. The samples being treated with conventional methods (75% ethanol vapor or ethanol soaking) or nothing were set as controls. According to the volume ratio of the HA aqueous solution and ethanol, the concentration of the ethanol used to immerse the samples was actually 86.65% (v/v). To find the optimal crosslinking time for the HA/EDC/NHS solution, the samples were treated with HA (0.05%)/EDC/NHS for different times (12 h, 24 h and 48 h). To find the optimal HA concentration, the samples were crosslinked with EDC/NHS solutions containing different amounts of HA.

## Characterization of the crosslinked nanofibrous matrices

The cross-linked matrices were rinsed with ultrapure water three times and vacuum dried for 24 h before use. The morphology of the matrices was characterized by SEM (TM-1000, Hitachi Ltd., Japan), with all samples being coated with gold film at 4 mA for 20 s before the measurements.

The mechanical properties of the samples were characterized using a universal material testing machine (H5K-S, Hounsfield, England) under the constant temperature and humidity conditions of 20 °C and 60% RH. The samples were cut into 10 mm × 50 mm portions and the average thickness of the nanofibrous mats was measured by a micrometer caliper three times. In this test, the distance of the two chucks was controlled at 30 mm before the start and the rate was 10 mm $min^{-1}$ throughout the whole process. The test was finished when the matrices broke under tensile force. Each sample was tested four to six times to get an average result.

The secondary structure of the samples was obtained by FTIR spectroscopy (Avatar380, USA) at room temperature in the wavenumber range of 4000 to 600 $cm^{-1}$.

The degree of crosslinking of the samples was determined using a TNBS assay which was described by Bubnis *et al.*[35] In brief, 3 mg samples crosslinked by HA (0.05%)/EDC/NHS for 12, 24 and 48 h were immersed in 2 ml of freshly prepared TNBS solution (1 ml of 4% (w/v) $NaHCO_3$ solution and 1 ml of 0.5% (w/v) TNBS in deionized water) and incubated at 40 °C for 2 h.

3 ml of 6 M HCl was added to terminate the reaction and the mixture was kept at 60 °C for 90 min. The resulting solutions were detected at 345 nm using a UV-VIS spectrophotometer. The degree of crosslinking was expressed as the loss of free amino groups after crosslinking and calculated as follows:

$$\text{Degree of crosslinking} = (A_0 - A_t)/A_0 \times 100\%$$

where $A_0$ is the absorbance of the SF nanofibrous mats without any treatment and $A_t$ is the absorbance of the SF nanofibrous matrices crosslinked for different amounts of time.

The post-spin treated materials were posted on a clean glass slide, droplets of water were injected into the surface of the material and the water contact angle of the samples was detected by Contact Angle Meter (OCA40, Datephysics, Germany). A 5 s video was obtained by the software and the angle of the samples was analyzed by Image J at the time point of 1 s. The average contact angles were obtained by measuring each sample three times.

### Cell culture and analysis

L929 cells were cultured in a DMEM medium (GIBCO, USA) supplemented with 10% fetal bovine serum (Invitrogen, USA), 1% penicillin/streptomycin (Invitrogen) was added and the cells were incubated in a humidified incubator at 37 °C, with 5% $CO_2$. The nanofibrous matrices were collected on circular glass cover slips (14 mm in diameter) for a cellular study. After treatment with 75% (v/v) ethanol vapor for 12 h, nanofiber-deposited cover slips were placed into a 24-well culture plate without further sterilization, and fixed with autoclaved stainless steel rings. Nanofiber-free coverslips were used as controls. L929 cells were seeded on the nanofibrous matrices at the same density of $1.0 \times 10^4$ cells per well.

L929 cells were cultured for 1, 3 and 5 days; the fresh medium was changed every two days. At a certain time point, the medium was sucked out and supplemented with 400 μl of serum-free DMEM containing 0.5 mg $ml^{-1}$ MTT in each well. After a 4 h incubation period, the medium was removed

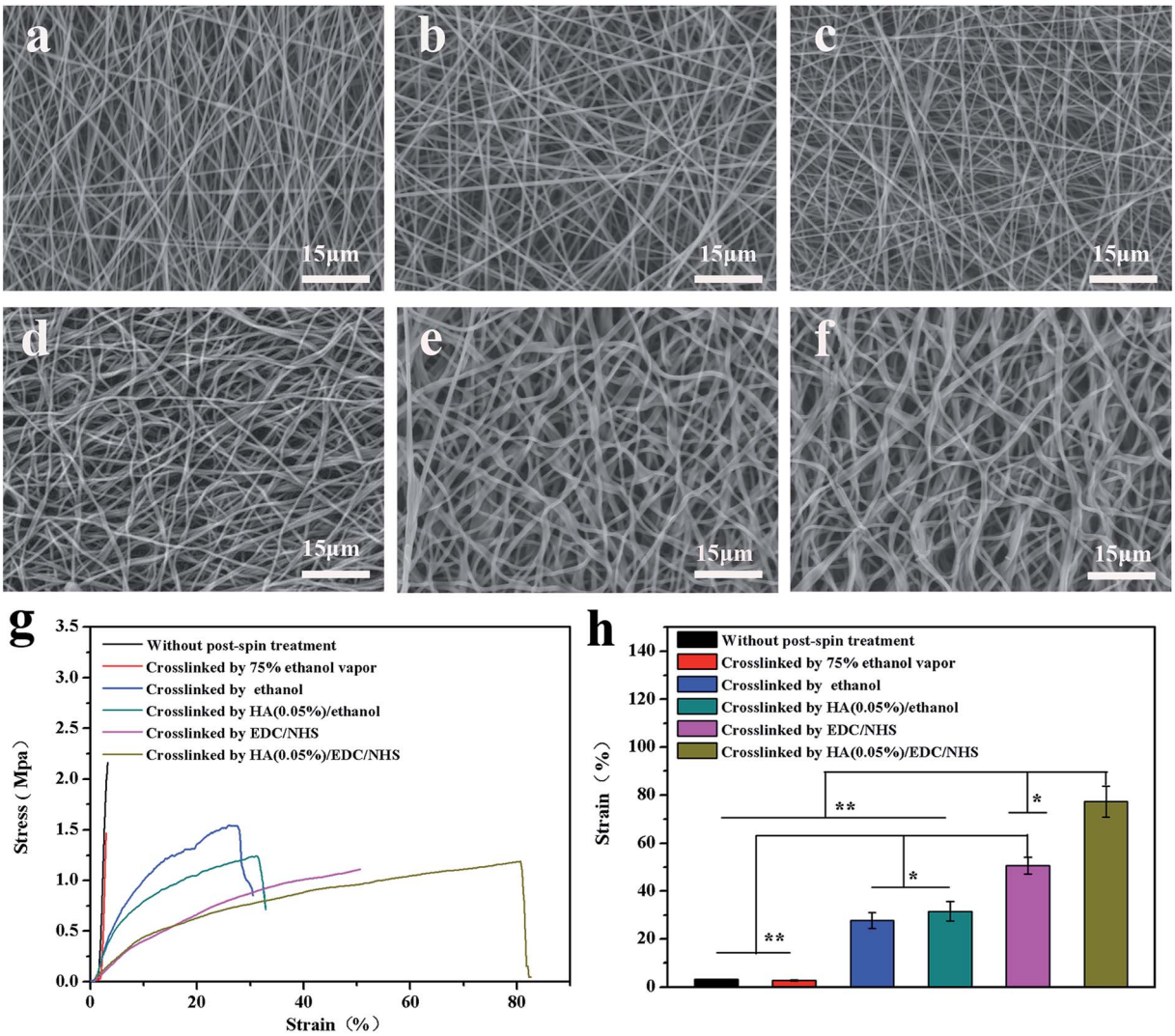


Fig. 1 SEM images and tensile performances of the SF nanofibers that were post-spin treated at room temperature for 24 h. (a) Without post-spin treatment; (b) crosslinked by 75% ethanol vapor; (c) crosslinked by ethanol; (d) crosslinked by HA (0.05%)/ethanol; (e) crosslinked by EDC/NHS; (f) crosslinked by HA (0.05%)/EDC/NHS; (g) stress–strain curves of (a–f); (h) the biggest strains of (a–f) (*$p < 0.05$, **$p < 0.01$).

 

completely and each well was supplemented with 400 μl of dimethylsulfoxide (DMSO). The 100 μl resulting solution was transferred to a 96 well plate and absorbance was detected at 492 nm using a microplate reader (Thermo, USA) after being shaken in a constant temperature shaker (Thermo, USA) at 37 °C for half an hour.

The medium in the 24 well plate was removed on the third day, 4% paraformaldehyde was used to fix the cells at 4 °C for 30 min and the cells were rinsed with PBS three times. The samples were then divided into two parts and treated respectively as follows: (1) dehydrated using 10%, 30%, 50%, 60%, 70%, 80%, 90%, 95% and 100% (v/v) ethanol, and then dried in a vacuum chamber. The morphology of the cells on the nanofibrous matrices was finally observed using SEM (Hitachi, Japan); (2) treated with 0.1% (v/v) Triton X-100 for 5 min and then treated with 2% (w/v) bovine serum albumin (BSA) for 30 min, the cells were then stained with DAPI for 15 min and Alexa Fluor®568 phalloidin for 30 min. A fluorescence microscope was finally used to capture an image of the cells.

### Statistical analysis

All experiments were conducted at least three times and the data were reported as the mean ± standard deviation (SD). Statistical analysis was performed using Student's *t*-test. In all statistical comparisons, a *p*-value of less than 0.05 was considered statistically significant. The error bars in the figures are the SDs of the data.

## Results and discussion

### Morphology and tensile properties of the crosslinked nanofibrous matrices

Smooth nanofibers with a relatively homogeneous morphology were obtained from the SF aqueous solution by electrospinning

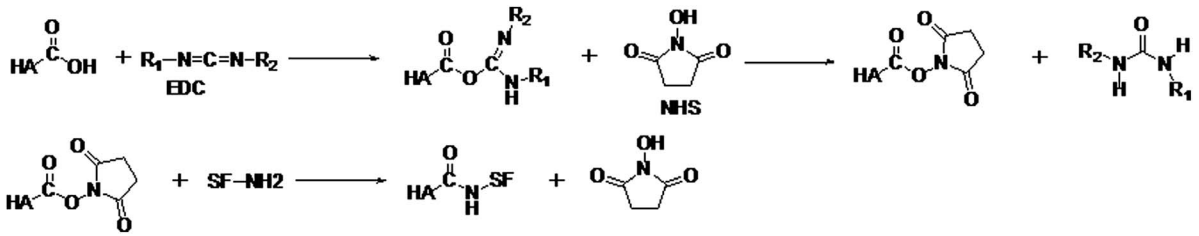


Fig. 2 Mechanism of the reactions for SF, HA and EDC/NHS.

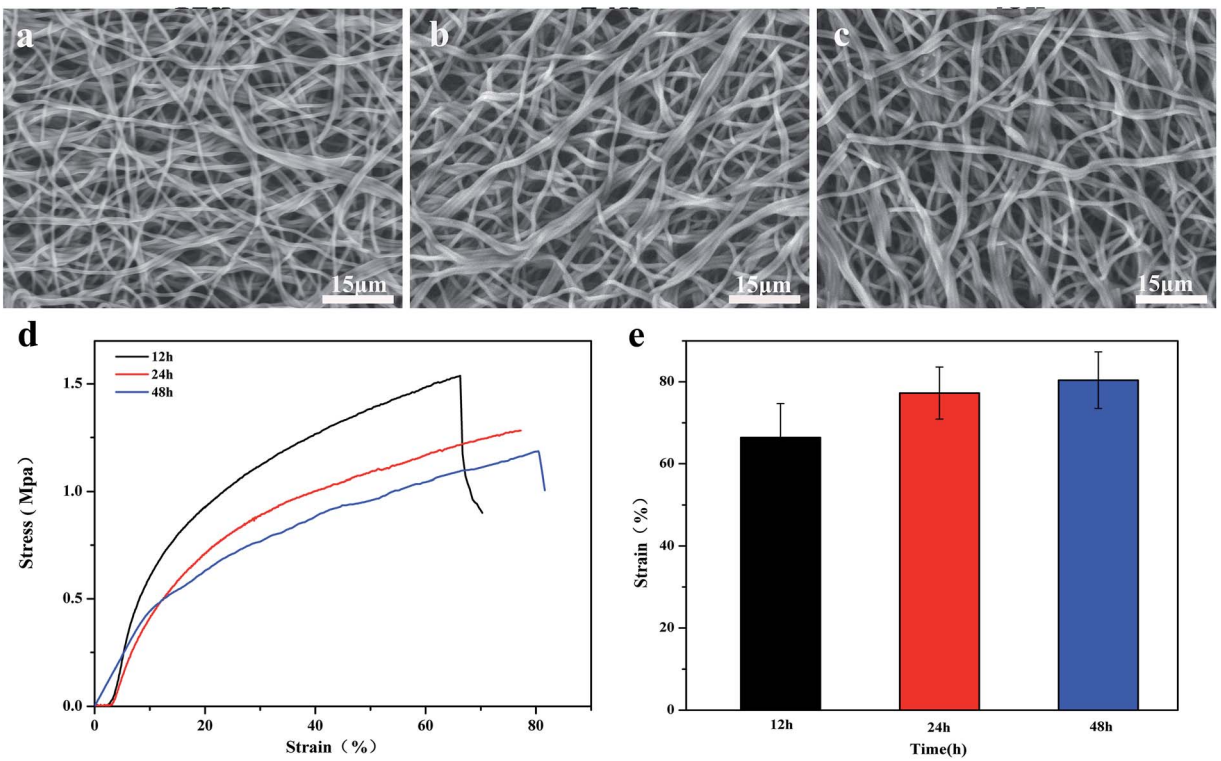


Fig. 3 SEM images and tensile properties of the SF nanofibrous matrices crosslinked by HA (0.05%)/EDC/NHS at room temperature for different times. (a) 12 h; (b) 24 h; (c) 48 h; (d) stress–strain curves of (a–c); (e) the biggest strains of (a–c).

and then crosslinked using different methods. The commonly used post-spin treatments of 75% (v/v) ethanol vapor or ethanol soaking were selected as controls, and the samples without any post treatment were set as the negative control. As shown in Fig. 1, the samples without post-spin treatment were easy to fracture with a 3.2% extension at break. For the samples with a treatment of 75% (v/v) ethanol vapor, the morphology and tensile performance of the nanofibers were similar to the negative control, without any improvement (Fig. 1a, b, g and h). The tensile performance of the nanofibers increased after being soaked in ethanol. However there was no significant difference between the ethanol and ethanol/HA groups, though some fibers had a bending morphology with the existence of HA (Fig. 1d, g and h). Almost all of the fibers started to bend and some fibers were bonded together after EDC/NHS-crosslinking (Fig. 1e) due to the interaction of EDC/NHS with the molecular chain of silk,[32] which in turn contributed to an improvement of the tensile properties of the nanofibers (extension at break increased up to 50%) (Fig. 1g and h). The morphology of the HA/EDC/NHS crosslinked samples changed more dramatically, with many fibers being bonded to each other (Fig. 1f), which may be due to the effect of the amidation reaction between HA, SF and EDC/NHS (Fig. 2), and the biggest strain for the fibers was increased up to 80% (Fig. 1g and h).

We firstly reported an improvement in the anti-hydration capacity of the electrospun SF nanofibers treated with 75% (v/v) ethanol vapor, which has obvious advantages in terms of safety and convenience as the materials can also be effectively sterilized at the same time.[19] However, treatment with 75% (v/v) ethanol vapor hardly improves the mechanical properties of SF nanofibers according to the data (Fig. 1g and h). Here, our data showed that the treatment of ethanol soaking did indeed significantly enhance the strain of the SF nanofibers, but the addition of HA had no apparent effect on the tensile performance. In contrast, the introduction of EDC/NHS or HA/EDC/NHS greatly enhanced the strain of the samples, and the

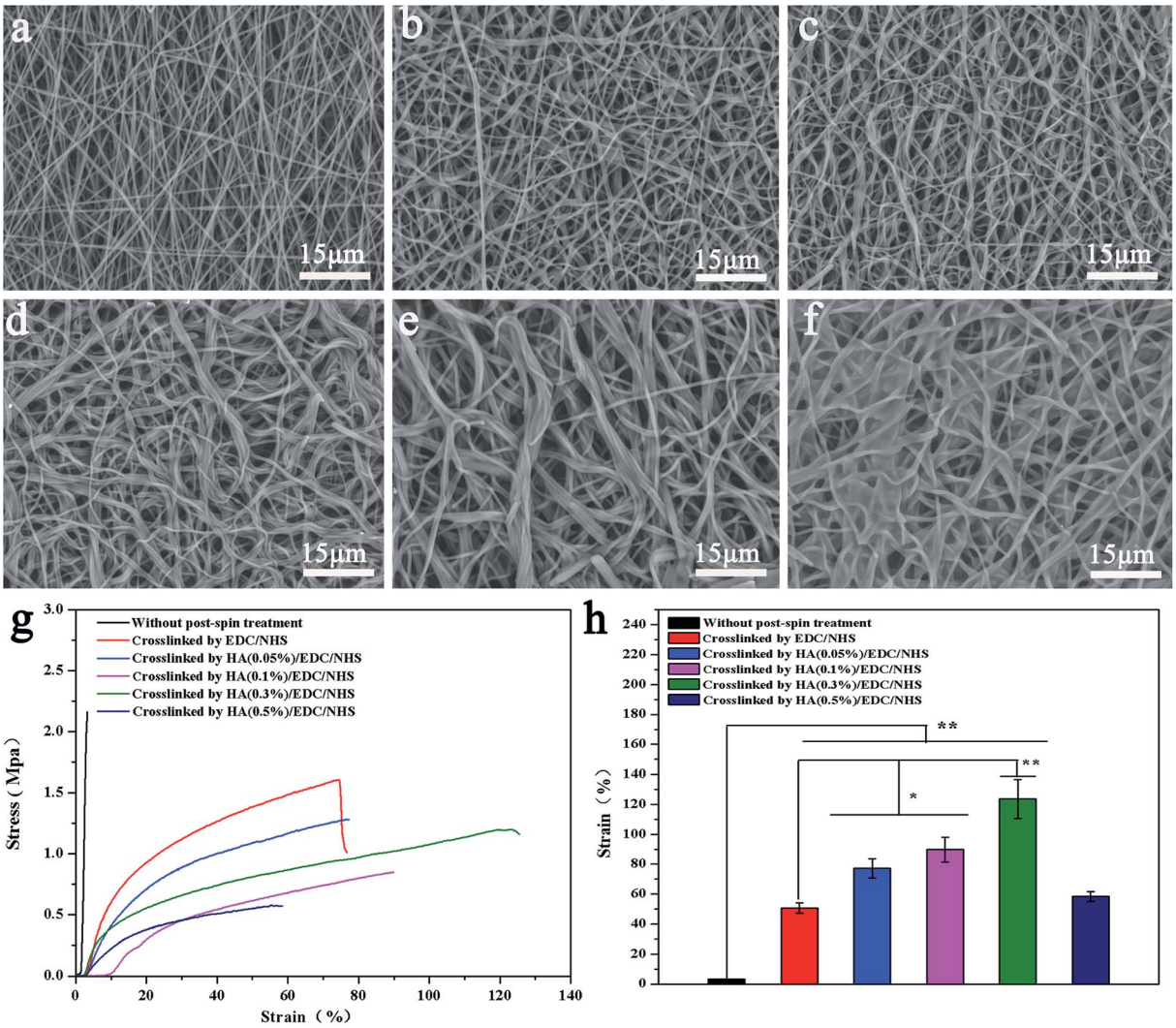


Fig. 4 SEM images and tensile performances of the SF nanofibers crosslinked by the HA/EDC/NHS solutions at room temperature for 24 h. (a) Without post-spin treatment; (b) crosslinked by EDC/NHS; (c) crosslinked by HA (0.05%)/EDC/NHS; (d) crosslinked by HA (0.1%)/EDC/NHS; (e) cross-linked by HA (0.3%)/EDC/NHS; (f) crosslinked by HA (0.5%)/EDC/NHS; (g) stress–strain curves of (a–f); (h) the biggest strains of (a–f) (*$p < 0.05$, **$p < 0.01$).

existence of HA further strengthened the crosslinking effect of EDC/NHS (Fig. 1g and h). These data demonstrated that HA/EDC/NHS-crosslinking is a better post-spin treatment for the electrospun SF nanofibers.

### The influence of crosslinking time on the tensile properties of the SF nanofibers

The SF nanofibrous matrices were crosslinked by the HA (0.05%)/EDC/NHS solution for 12, 24 and 48 hours at room temperature to determine an optimal crosslinking time. More nanofibers became bent with an increase in the crosslinking time from 12 h to 24 h (Fig. 3a and b), and the biggest strain extended from 70.3% to 77.25% (Fig. 3d and e). However, there was no significant difference between 24 h and 48 h (Fig. 3c–e). The degree of crosslinking for the samples treated with the HA (0.5%)/EDC/NHS solution for 12, 24 and 48 h was 23.6%, 30% and 31.9% respectively. These results suggested that the crosslinking reaction was substantially completed within 24 hours. Therefore, 24 h is the optimal crosslinking time for SF nanofibers treated with the HA/EDC/NHS solution.

### The influence of HA concentration on the tensile properties of the SF nanofibers

According to the above data, the tensile properties of the SF nanofibers can be evidently improved through the crosslinking of EDC/NHS, and this effect can be further strengthened by HA. Thus, it is necessary to find the optimal concentration of HA, at which the tensile properties of the SF nanofibers would have the maximum increase. The nanofibers were treated for 24 h with 50 ml of the EDC/NHS solutions containing different amounts of HA (0, 0.05%, 0.1%, 0.3% and 0.5% (w/v)) at room temperature. As shown in Fig. 4a–e, more and more nanofibers started to bend and became entangled with each other, forming bundles as the HA concentration increased from 0 to 0.3% (w/v). The strain performance of the treated samples also increased with similar trends (Fig. 4f and h). However, when the concentration of HA increased up to 0.5% (w/v), the samples looked very different from the others: the bundled fibers decreased and the pores were covered by something (probably HA), and the strain performance was reduced to the levels of those without HA. Obviously, too much HA is detrimental to the crosslinking effect of HA/EDC/NHS from the perspective of improving the tensile properties of the SF nanofibers, so the optimal concentration of HA is 0.3% (w/v), at which the biggest strain can reach up to 120%. Thus, a HA concentration of 0.5% (w/v) wasn't used in the following study.

In recent years, engineering scaffolds based on the electrospun SF nanofibers has attracted much attention due to the unique merits of SF and its ability to mimic the natural ECM. However, the SF nanofibers are very fragile due to the damage of the nanoscale structure of native silk during the dissolution and regeneration process,[9,36] which greatly restricts their practical application in tissue engineering. The commonly used methods of post-spin treatment such as ethanol or water vapor,[19,25] have no effect on the mechanical properties of the SF nanofibers. Fan *et al.* reported a special post-spin approach which significantly improved the strength performance of the electrospun SF microfibers,[37] but their method involved a complex process and the fiber diameter was more than 1000 nm which is not conducive to mimicking the ECM. A new SF nanofibrous film from directly dissolving silk in a $CaCl_2$–formic acid solution was recently reported with robust mechanical performance.[38] Yet, they used formic acid which is eco-unfriendly and has potential hazards for cells. Besides, their method is not applicable to the preparation of three-dimensional scaffolds. Although the introduction of HA was reported to enhance the strain performance of the SF films up to 138% when the content of HA was increased to 5%,[27,28] these SF/HA films have a dense and imporous structure which can be adverse to cell growth.

We have been focusing on the fabrication and application of green electrospun SF nanofibers in consideration of safety and

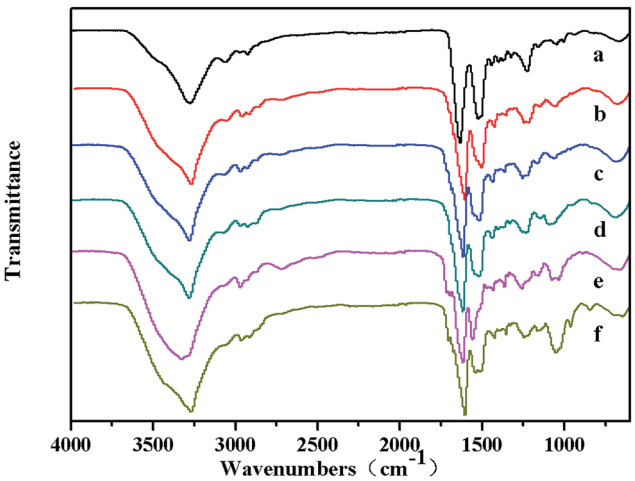


Fig. 5 FTIR spectra of the SF nanofibrous matrices, (a) without post-spin treatment; (b) crosslinked by ethanol soaking; (c) crosslinked by EDC/NHS; (d) crosslinked by HA (0.05%)/EDC/NHS; (e) crosslinked by HA (0.1%)/EDC/NHS; (f) crosslinked by HA (0.3%)/EDC/NHS.

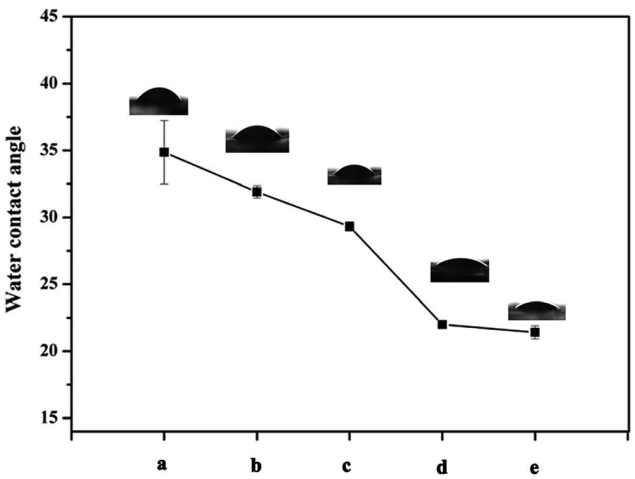


Fig. 6 Water contact angle of the SF nanofibrous matrices, (a) without crosslinking; (b) crosslinked by EDC/NHS; (c) crosslinked by HA (0.05%)/EDC/NHS; (d) crosslinked by HA (0.1%)/EDC/NHS; (e) crosslinked by HA (0.3%)/EDC/NHS.

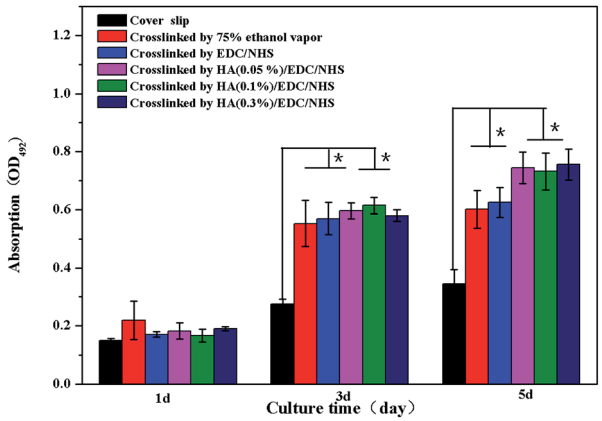


Fig. 7 The viability of L929 cells grown on different substrates (*$p < 0.05$).

convenience.[4,12,13] The most troublesome and urgent matter to address for the application of green electrospun SF nanofibers is the poor mechanical performance. Here, we demonstrated that the flexibility of the SF nanofibrous matrices increased dramatically after crosslinking with HA/EDC/NHS, though the strength showed little improvement. EDC is a water soluble and non-cytotoxic carbodiimide which can crosslink an amino linkage as the coupling agent, and NHS is usually used as a stabilizer to improve the crosslinking efficiency of EDC.[39] It was demonstrated that EDC/NHS concentrations that are less than 0.5 M can be safely used in biomaterials.[40] HA, a natural biological macromolecule, is involved in various physiological processes as a major component of the ECM, and can be activated and modified by EDC/NHS.[33,34] Thus, HA/EDC/NHS crosslinking is actually a safe and eco-friendly process and could be beneficial to the biological function of the SF nanofibers. Our data suggested a good potential for the HA/EDC/NHS-crosslinked SF nanofibrous matrices to serve in the biomedical field, especially in skin tissue regeneration, since the SF nanofibers offer great benefits for skin tissues.[4,12,13]

## FTIR analysis

FTIR spectroscopy was used to study the structural characteristics of the HA/EDC/NHS-crosslinked SF nanofibrous matrices. As shown in Fig. 5, after crosslinking, the characteristic absorption bands of the SF at 1630 $cm^{-1}$ (amide I), 1520 $cm^{-1}$ (amide II) and 1234 $cm^{-1}$ (amide III) shifted to 1600 $cm^{-1}$, 1540 $cm^{-1}$ and 1200 $cm^{-1}$ respectively, which indicated that the conformation of silk changed from silk I into silk II[19] (Fig. 5b–f). Indeed, the water-resistance of the as-crosslinked SF nanofibrous matrices was greatly enhanced, to such an extent that the structure of the matrices still kept intact after being soaked in PBS for 7 days. With an increased concentration of HA, the absorption peak at 3250–3300 $cm^{-1}$ broadened, and a new peak at 1000–1300 $cm^{-1}$ appeared and was enhanced, which demonstrated that HA was attached to the SF nanofibers by covalent crosslinks (Fig. 5d–f).

## Hydrophilic–hydrophobic properties

Water contact angles were detected in order to study the hydrophilic–hydrophobic properties of the SF nanofibrous matrices. Before crosslinking, the water contact angle of the SF nanofibrous matrices was 34.9°, while the angles became smaller after crosslinking (Fig. 6). The water contact angles of the crosslinked samples then gradually decreased with an increase in HA content, which indicated that HA helped to

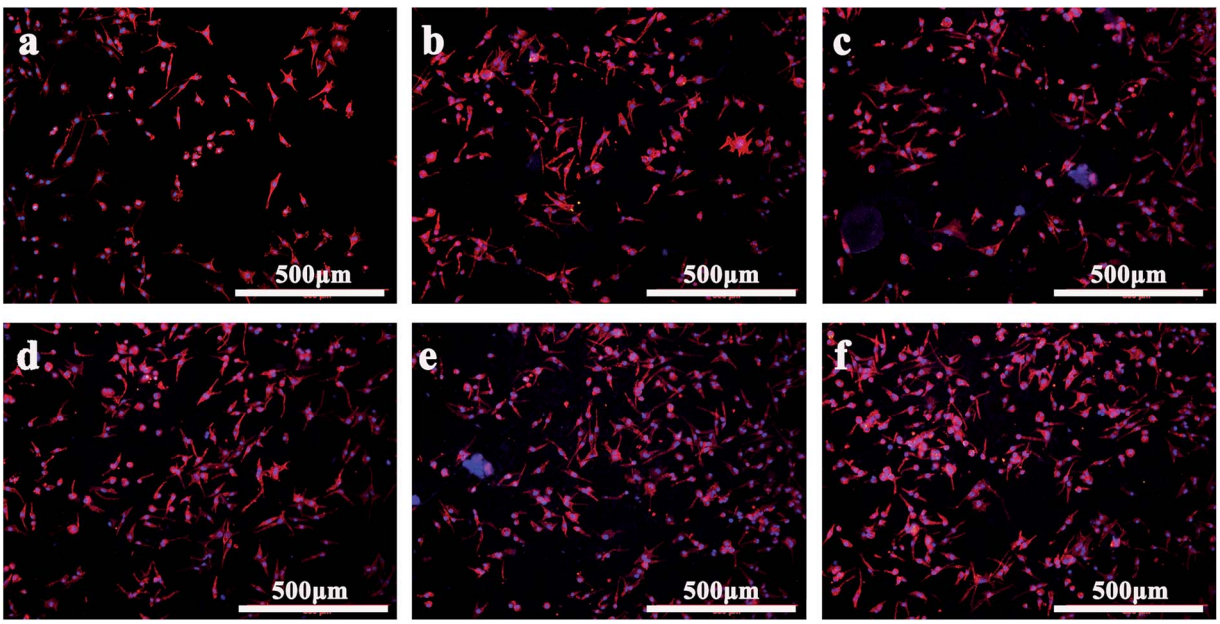


Fig. 8 Fluorescence images of L929 cells cultured on different substrates, (a) coverslip; (b) 75% ethanol vapor crosslinked SF nanofibrous matrices; (c) EDC/NHS crosslinked SF nanofibrous matrices; (d) HA (0.05%)/EDC/NHS crosslinked SF nanofibrous matrices; (e) HA (0.1%)/EDC/NHS crosslinked SF nanofibrous matrices; (f) HA (0.3%)/EDC/NHS crosslinked SF nanofibrous matrices.

 

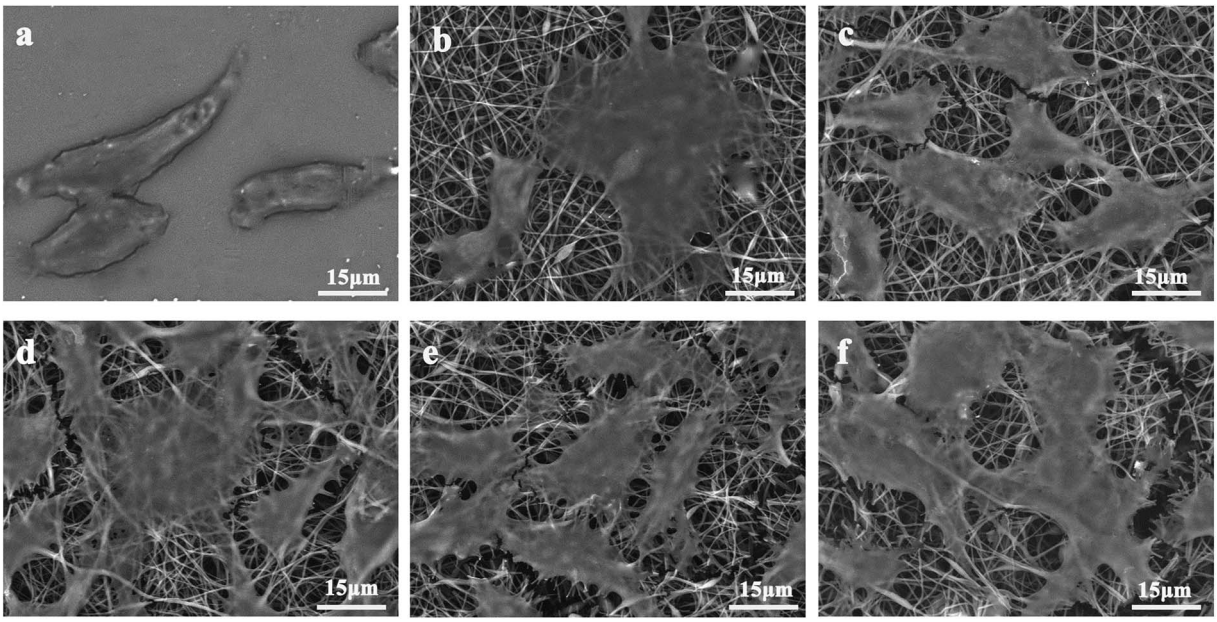


Fig. 9 SEM images of L929 cells cultured on different substrates, (a) cover slip; (b) 75% ethanol vapor crosslinked SF nanofibrous matrices; (c) EDC/NHS crosslinked SF nanofibrous matrices; (d) HA (0.05%)/EDC/NHS crosslinked SF nanofibrous matrices; (e) HA (0.1%)/EDC/NHS crosslinked SF nanofibrous matrices; (f) HA (0.3%)/EDC/NHS crosslinked SF nanofibrous matrices.

enhance the hydrophilicity of the SF nanofibers. The increase of hydrophilicity is helpful for the electrospun nanofibrous scaffolds to resist non-specific protein adsorption and helps to enhance cell infiltration.[41]

### Cytocompatibility of the crosslinked nanofibrous matrices

An MTT assay was used to measure the proliferation of L929 cells grown on the crosslinked SF nanofibrous matrices by detecting the absorbance at 492 nm. As shown in Fig. 7, the cells grown on the nanofibrous matrices proliferated faster than those on the glass, and there was no difference among the various crosslinked SF nanofibrous matrices, which is confirmed by the fluorescence microscopy data (Fig. 8). These results indicated that HA/EDC/NHS-crosslinked SF nanofibrous matrices have good cytocompatibility.

The morphology of the L929 cells grown on HA/EDC/NHS-crosslinked SF nanofibrous matrices was observed by SEM, as shown in Fig. 9. L929 cells cultured on the SF nanofibrous matrices presented a much better morphology than those on coverslips. The cells spread widely and were bridged to each other on the nanofibrous matrices, which could be beneficial for signal transduction among the cells and in turn promoting cell proliferation.[42] It has also been demonstrated that the HA-based scaffolds can activate TGF-β1/MMPs signalling pathways through interaction with the cell surface receptor CD44, which is conducive to cell migration.[41]

## Conclusions

In summary, the tensile properties of the green electrospun SF nanofibrous matrices were significantly improved through a simple and eco-friendly process of HA/EDC/NHS-crosslinking. The appropriate crosslinking time was 24 h and the optimal concentration of HA was 0.3% (w/v), at which the biggest strain of the nanofibrous matrices increased up to 120%. After crosslinking, the nanofibers entangled into bundles which greatly contributed to the enhancement of strain. The introduction of HA also increased the hydrophilicity of the matrices, which is conducive to the resistance of non-specific protein adsorption. Moreover, the HA/EDC/NHS-crosslinked SF nanofibrous matrices showed good cytocompatibility. The present study indicates that HA/EDC/NHS-crosslinking is a good post-spin treatment for green electrospun SF nanofibers and as-treated SF nanofibrous matrices could find a promising application in biomedical fields, especially in soft tissue regeneration.

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