

Tough Biopolymer IPN Hydrogel Fibers by Biezymatic Crosslinking Approach^{*}

Xiao-jiu Liu, Ye-min Zhang and Xin-song Li^{**}

School of Chemistry and Chemical Engineering, Southeast University, Nanjing 210018, China

Abstract In this report, biezymatic crosslinking approach was applied to prepare biopolymer hydrogel fibers composed of gelatin and chitosan with an interpenetrating polymer network (IPN) structure. The IPN biopolymer hydrogel fibers were prepared by wet spinning while microbial transglutaminase (mTG) catalyzed the formation of one network of gelatin and horseradish peroxidase (HRP) in the presence of H₂O₂ induced another network of chitosan grafted with phloretic acid (chitosan-PA) intertwining with the former. The mechanical performances of the hydrogel fibers were measured by an electronic single fiber strength tester. It was found that the mechanical properties of the gelatin/chitosan IPN hydrogel fibers had a significant improvement with the increase of the concentration of gelatin and chitosan, and the IPN fiber has the highest tension of 38.6 cN and elongation of 387.2%. Accelerated degradation in the presence of papin indicated that the gelatin/chitosan-PA IPN hydrogel fibers own controllable degradability. In addition, biological evaluation indicated the IPN hydrogel fibers can support cell adhesion and proliferation. Moreover, hand weaving trail showed the knittability of the IPN hydrogel fibers. Therefore, the gelatin/chitosan IPN hydrogel fibers prepared by biezymatic crosslinking approach possess excellent biocompatibility and mechanical strength, which may be desirable candidates for engineering tissue scaffolds.

Keywords: Hydrogel fiber; Biezymatic crosslinking; Gelatin; Interpenetrating polymer network; Mechanical strength.

INTRODUCTION

Hydrogel fibers composed of natural polymers have high surface area and porosity, which can give full play to the excellent biological properties of natural polymer materials, have great potential to exploit the wider research possibilities in the development of tissue engineering scaffolds^[1–4]. The fibers can be woven, knit, braided, continuous randomly aligned (2D or 3D), staple fibers randomly aligned, and can be designed to have extremely high porosity for cellular infiltration, and exhibit good mechanical properties and suitable compliance for handling and conformability to varying tissue and organ shapes^[1, 5]. But most of hydrogel fibers made from natural polymers lack mechanical strength limiting their applications as biomedical materials.

Up to now, several methods have been developed to improve the mechanical strength of hydrogel fibers^[6–8]. Meyer prepared collagen fibers using chemical cross-linking^[9]. Although chemical crosslinking can effectively enhance the mechanical strength of the hydrogels, but the residues of crosslinkers or initiators may bring about cytotoxic side-effects even after thorough purification^[10, 11]. Physical crosslinking is a mild process without using chemical substances, but the mechanical properties of the hydrogels are insufficient, and the crosslinking points are easy to disintegrate in biological fluids^[12–15]. Therefore, to prepare hydrogel fibers with toughness and biocompatibility remains a challenge.

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^{**} Corresponding author: Xin-song Li (李新松), E-mail: lixs@seu.edu.cn

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Enzymatic crosslinking, completely devoid of potentially cytotoxic small molecule crosslinkers, is a biocompatible approach in the development of hydrogels^[16, 17]. Unfortunately, single enzyme-crosslinked hydrogels have poor mechanical strength and rapid degradation, whose application is often subjected to some restrictions^[18–20]. They were usually applied as injectable biomaterials, such as, scaffolds or tissue fillers. In this report, bienzymatic crosslinking was applied to prepare biopolymer based IPN hydrogel fibers to improve mechanical strength while possessing favourable biocompatibility. The IPN hydrogel fibers were prepared by wet spinning using microbial transglutaminase (mTG) to catalyze the formation of gelatin network and horseradish peroxidase (HRP) to induce the formation of chitosan network. The mechanical strength, swelling ratio, degradation, and cytocompatibility of the IPN hydrogel fibers were further examined in detail. Finally, the knittability of the fibers was tested.

MATERIALS AND METHODS

Materials

Water-soluble chitosan (8 kDa, 90% DD) was purchased from Shunbo Biological Technology Research Institute Co., Ltd (Qingdao, China). Gelatin (type A), horseradish peroxidase (HRP, 250 U·mg⁻¹), hydrogen peroxide (H₂O₂, 30% wt) and papain (6000 U·mg⁻¹) were obtained from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Microbial transglutaminase (mTG, 1000 U·g⁻¹) was a gift from Dongsheng Food Science and Technology Co., Ltd (Taixin, China).

Fetal bovine serum (FBS), Dulbecco's Modified Eagle's Medium (DMEM), 3-(4,5-dimethylthiazol-2-yl)-2, penicillin-streptomycin solution and 5-diphenyltetrazolium bromide (MTT) were purchased from Invitrogen Co. (Carlsbad, CA). L929 mouse fibroblast cells were a gift from the Public Health of Southeast University. All reagents were of analytical grade.

Preparation of IPN Hydrogel Fibers

Chitosan was grafted with phloretic acid (chitosan-PA, degree of substitution 29.3%) used as a substrate of horseradish peroxidase (HRP) as described in our previous report^[21]. The gelatin/chitosan-PA IPN biohydrogel fibers were prepared by wet spinning as illustrated in Fig. 1. The compositions of spinning dopes are listed in Table 1. Briefly, gelatin was dissolved in chitosan-PA solution to obtain a polymer solution mixture at 37 °C followed by HRP addition with the final concentration of 5 U·mL⁻¹. To prepare IPN hydrogel fibers, the spinning dope of gelatin/chitosan-PA and HRP was spun at a speed of 10 mL·h⁻¹ into aqueous coagulant bath with the mTG concentration of 15 U·mL⁻¹ and H₂O₂ of 0.8 mmol·L⁻¹ through a stainless steel spinneret with the diameter of 0.9 mm, and the fibers were rinsed quickly with distilled water to remove uncrosslinked biopolymers on the surface. After that, the resultant fibers were kept in an oven at 40 °C for 30 min to complete the mTG enzymatically crosslinking. Then, the diameter of these fibers was measured by a micrometer caliper, which is in the range of (0.9 ± 0.1) mm.

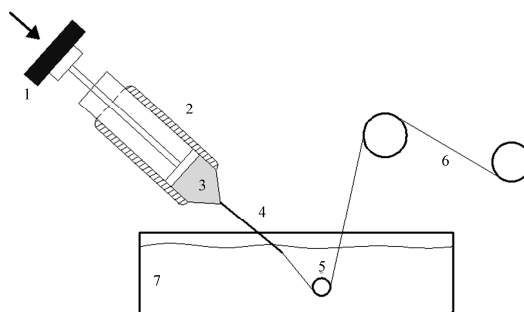


Fig. 1 Schematic diagram of wet spinning procedure: (1) metering pump, (2) attenuator, (3) spinning dope, (4) spinneret, (5) draw-off roller, (6) hydrogel fiber and (7) coagulation bath

Table 1. Composition of gelatin/chitosan-PA hydrogel fibers

Sample	Gelatin (mg·mL ⁻¹)	Chitosan-PA (mg·mL ⁻¹)	mTG (U·mL ⁻¹)	HRP (U·mL ⁻¹)	H ₂ O ₂ (mmol·L ⁻¹)
G1	100	0	15	0	0
C1	0	10	15	5	0.8
IPN					
G10C1	100	10	15	5	0.8
G10C1.5	100	15	X [#]	5	0.8
G10C2	100	20	15	5	0.8
G12.5C1	125	10	15	5	0.8
G12.5C1.5	125	15	15	5	0.8
G12.5C2	125	20	15	5	0.8
G15C1.5	150	15	X [#]	5	0.8
G15C2	150	20	15	5	0.8
G17.5C1	175	10	15	5	0.8
G17.5C1.5	175	15	15	5	0.8
G17.5C2	175	20	15	5	0.8
G20C1.5	200	15	X [#]	5	0.8
G20C2	200	20	15	5	0.8

X[#]: Different concentrations of mTG (10 U·mL⁻¹, 15 U·mL⁻¹, 20 U·mL⁻¹, 30 U·mL⁻¹) were used.

Mechanical Test

Mechanical properties of the IPN hydrogel fibers were characterized by an electronic single fiber strength tester (LLY-06B, Laizhou Electronic Instrument Co., Ltd, Jinan, China) at room temperature. Hydrogel fiber with the length of 6 cm was subjected to tensile test at a strain rate of 20 mm·min⁻¹. All samples were measured in triplicates.

Swelling Test

Gelatin/chitosan-PA hydrogel fibers were incubated in distilled water at 37 °C for 24 h to reach swelling equilibrium. After removal of excess surface water, the swollen weight (W_s) of each hydrogel fiber was recorded. Then the samples were lyophilized and the dry weight (W_d) of each sample was weighed. The swelling ratio was calculated using the following equation:

$$\text{Swelling ratio} = \frac{W_s - W_d}{W_d}$$

All samples were tested in triplicates for each group.

In vitro Enzymatic Degradation

To test enzymatic degradation of the IPN fibers, the lyophilized hydrogel fibers (W_0) were equilibrated in PBS. Then, the samples were immersed in PBS solution containing papain (90 U·mL⁻¹) at 37 °C with shaking at 100 r·min⁻¹ for 3 h, 6 h, 9 h and 12 h respectively. At each time point, the gels were washed with distilled water, and then lyophilized to measure the weight (W_t). The fractional mass remaining was expressed using the following equation:

$$\text{Fractional mass remaining (\%)} = \frac{W_t}{W_0} \times 100\%$$

Each group was performed in triplicates.

In vitro Cytotoxicity

The cytotoxicity of the gelatin/chitosan IPN hydrogel fibers was evaluated by an extraction test according to ISO 10993-5 Standard^[22]. Briefly, sterilized hydrogel fibers were extracted using DMEM containing 10% FBS at an extraction ratio of 1 cm²·mL⁻¹ at 37 °C for 24 h. A 100 μL media suspension containing total of

10000 cells·well⁻¹ and 100 μ L of extract solution were plated into each well of the 96-well plate. As a blank group, 100 μ L media suspension and 100 μ L of complete medium were used. Both the sample and blank group were incubated at 37 °C in a 5% CO₂ humidified incubator. On the 1st, 3rd, and 5th days, 20 μ L of MTT (5 mg·mL⁻¹) solution was added into each well for 4 h to allow formation of formazan crystal. Subsequently, after removal of the supernatant, 150 μ L of DMSO was added to dissolve formazan crystals, and the absorbance was measured at 490 nm using an ELISA reader (Bio-Rad 860, Richmond, CA). The cell relative growth rate (RGR) was calculated as a percentage of the sample group to the blank group. All data were obtained from an average of six samples.

Cell Seeding and Imaging

L929 mouse fibroblasts were inoculated onto fibers surface for observation of cells' morphology on the samples. After 75% alcohol sterilization for 15 min, the hydrogel fibers were rinsed with sterile PBS and then incubated in DMEM for 4 h in 6-well plate. L929 cells were seeded onto the hydrogel scaffolds with a cell density of 1×10^5 cells·cm⁻² and incubated in a humidified atmosphere (37 °C, 5% CO₂) for 5 days. Cell morphology was observed by an optical microscope (BDS200, Zhengzhou, China). After 5 days, the cell-seeded fibers were fixed with 2.5% glutaraldehyde solution at room temperature for 2 h, and then rinsed with PBS three times and dehydrated in gradient ethanol series (25%, 50%, 70%, 85%, 95%, and 100%). Following that, the samples were freeze-dried, and the morphologies of cells on the fibers were observed under a scanning electron microscope (JSM-6360, JEOL, Japan) after gold sputter-coating.

RESULTS AND DISCUSSION

Gelatin/chitosan biohydrogels fibers were prepared using wet spinning (Fig. 1) by bienzymatic crosslinking of TG and HRP. Microbial transglutaminase (mTG) can catalyze the chemical reaction between glutamine and lysine residues on adjacent gelatin chains, thus providing amide bonds that contribute to form gelatin three-dimensional network^[23, 24]. After grafting with phloretic acid (PA) chitosan can be crosslinked by an HRP-mediated oxidative reaction of PA. Therefore, both gelatin and chitosan formed crosslinked networks independent of each other in the presence of both mTG and HRP as described in our previous report^[21], resulting in an IPN hydrogel fiber.

The spinning dope composed of gelatin, chitosan-PA and HRP was extruded and treated using mTG and H₂O₂ mixture as a coagulation bath. A series of gelatin/chitosan-PA solutions with different compositions as shown in Table 1 were used to test their spinnability. It was found that pure gelatin and pure chitosan-PA can't be made into fiber, and the spinning dope can be spun to form fibers when the concentration of gelatin is more than 100 mg·mL⁻¹ and the concentration of chitosan-PA more than 10 mg·mL⁻¹. The obtained hydrogel fibers have a chitosan-PA crosslinked network intertwined with another gelatin network, resulting in a mechanically reinforced and strengthened hydrogel system. After that, the physicochemical and biological properties of the gelatin/chitosan-PA IPN hydrogel fibers were investigated in detail as follows.

Mechanical Properties

The mechanical properties of gelatin/chitosan-PA IPN hydrogel fibers with the compositions as shown in Table 1 were measured by a single fiber tensile tester at room temperature. IPN fiber G10C1 was so weak that it couldn't be handled to test mechanical strength. Figure 2 shows the summary of the tension and elongation of the hydrogel fibers. It was revealed that the tension of IPN hydrogel fibers was increased with the increase of gelatin or chitosan-PA content.

As indicated in Fig. 2(a), increasing the concentration of gelatin from 10% g/mL to 20% g/mL resulted in significant raises of tensile strength and elongation at break. The tension of gelatin/chitosan IPN hydrogel fiber G20C1.5 reached 29.51 cN which was 3.44-fold higher than that of G10C1.5 fiber, and the tension of G20C2 was 38.56 cN 2.66-fold higher than that of G10C2. Apparently, the gelatin/chitosan-PA IPN fibers show the increase of tension and elongation when gelatin concentration increases. It can be attributed that gelatin was covalently crosslinked by TG-mediated reaction forming crosslinked network, and the increase of gelatin

concentration results in higher crosslinking density.

As the chitosan-PA concentration increased from 1% g/mL to 2% g/mL, in the group of G12.5C the tension of IPN fiber increased by 2.71-fold (6.46 cN versus 17.52 cN), and the elongation by more than 1.51-fold (193.9% versus 294.5%). In the group of G17.5C the tension of IPN fiber increased by 2.42-fold (13.35 cN versus 32.36 cN), and the elongation by more than 1.23-fold (306.4% versus 376.9%). Obviously, the mechanical strength of the IPN hydrogels was improved with the increase of chitosan-PA concentration, which can be ascribed to the raise of crosslinking density of chitosan. The gelatin/chitosan IPN fiber G20C2 having the highest tension of 38.6 cN and elongation of 387.2% is so tough that it should withstand further handling and post processing.

The effect of mTG concentration on the tensile strength of gelatin/chitosan IPN hydrogel fibers was checked further. As indicated in Fig. 3, increasing mTG concentration from 10 U·mL⁻¹ to 15 U·mL⁻¹ results in a slight elevation of tension. However, there is no significant change of strength when the concentration of mTG increases from 15 U·mL⁻¹ to 30 U·mL⁻¹. It means that the concentration of mTG in this range has no significant effect on the mechanical strength of IPN gel fibers because of complete crosslinking. So, the optimal concentration of mTG which can mediate a complete crosslinking of gelatin is about 15 U·mL⁻¹ in aqueous coagulant bath.

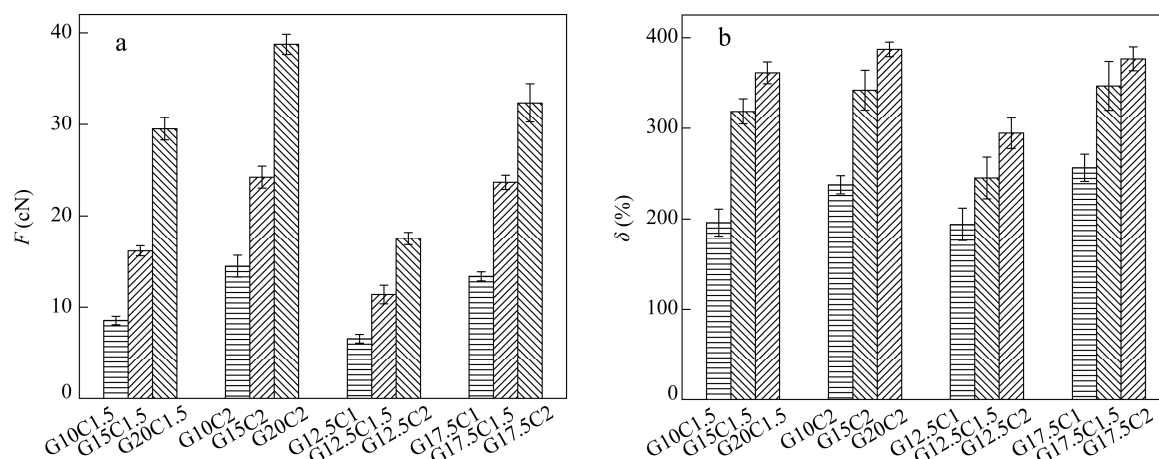


Fig. 2 Tension (a) and elongation (b) of gelatin/chitosan-PA IPN hydrogel fibers (The composition of the fibers is listed in Table 1.)

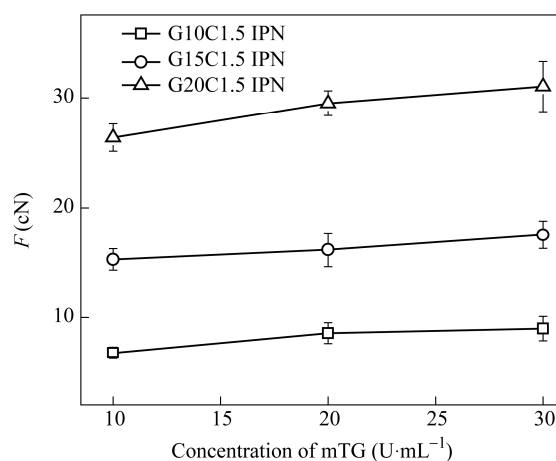


Fig. 3 Effect of mTG concentration on the tension of gelatin/chitosan-PA IPN hydrogel fibers G10C1.5, G15C1.5 and G20C1.5

Swelling Properties

The swelling behavior of gelatin/chitosan IPN hydrogel fibers was measured by gravimetric method and the results are given in Fig. 4. It was shown that gelatin/chitosan IPN hydrogel fibers have a significant decreasing of swelling ratio with the increase of chitosan-PA content. It can be ascribed that the formation of IPN network was more efficient at higher chitosan-PA concentrations, resulting in higher crosslinking density, which hindered the penetration of water in the hydrogel fibers. Besides, as gelatin concentration increased from 10% g/mL (G10C1.5) to 20% g/mL (G20C1.5), the swelling ratio of the IPN hydrogel fibers has a trend of slight increasing. This may be attributed to the gelatin forming hydrogen bonds with water molecules to absorb more water.

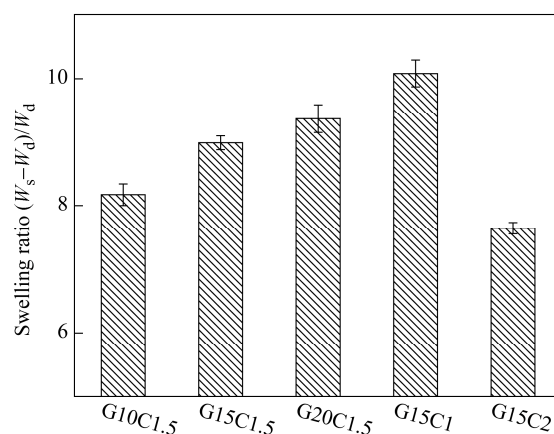


Fig. 4 Swelling ratio of gelatin/chitosan-PA IPN hydrogel fibers after incubating in distilled water for 24 h at 37 °C

Enzymatic Degradation

Biodegradability is an important indicator to evaluate the material whether has potential applications in tissue engineering scaffolds. The degradation property of gelatin/chitosan-PA IPN hydrogel fibers was characterized by measuring the weight change of fibers in PBS containing 90 U·mL⁻¹ papain. The data in Fig. 5 indicated that the degradation rate of IPN hydrogel fibers decreased with increasing chitosan-PA and gelatin concentrations. After 12 h incubation, the fractional mass remainings of G10C1, G12.5C1, G15C1, G15C1.5 and G15C2 fibers were 0, 14.5%, 21.8%, 28.3% and 39.7%, respectively. In general, the degradation behavior of the hydrogel fibers was

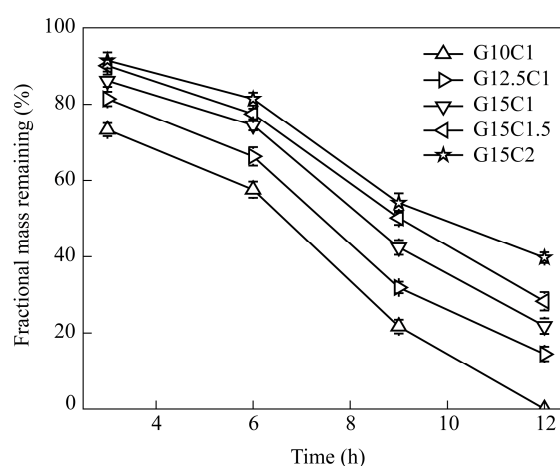


Fig. 5 Degradation profiles of gelatin/chitosan-PA IPN hydrogel fibers in PBS solution containing 90 U·mL⁻¹ papain at 37 °C

dependent on their cross-linking density. The result indicated that the IPN hydrogel fibers had higher degree of crosslinking with increasing concentration of gelatin and chitosan-PA. The higher concentration of biopolymers resulted in more stable and dense interpenetrating network, and thus the sites of biopolymers were more difficult to be approached and attacked by papain. Apparently, this kind of gelatin/chitosan-PA IPN hydrogel fibers owns controllable degradability. It makes the hydrogel IPN fibers gain good potential applications in tissue engineering scaffolds.

In vitro Cytotoxicity

In vitro cytotoxicity of the gelatin/chitosan-PA IPN hydrogel fibers was assessed using L929 cells incubated with extract solution for 1, 3 and 5 days. As shown in Fig. 6, the quantitative assessment indicated that all of the fibers were above 90%. The results indicated that the IPN hydrogel fibers are non-cytotoxic and would be candidate biomaterials of tissue engineering scaffolds.

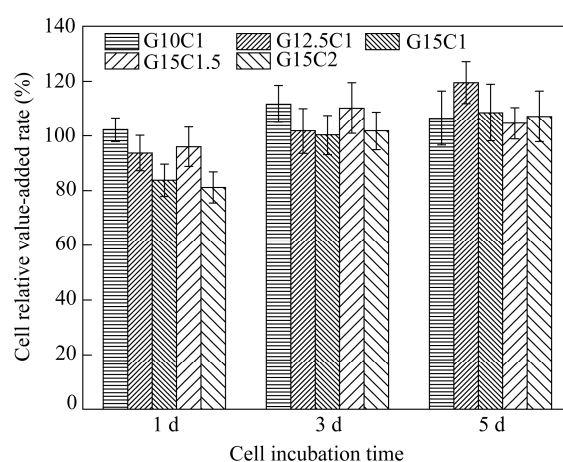


Fig. 6 Cytotoxicity of extracts of gelatin/chitosan-PA IPN hydrogel fibers with the composition as shown in Table 1 in comparison with blank group (cells with complete medium alone)

Cell Seeding and Imaging

Cell adhesion as the origin of cell proliferation is an important deciding factor for tissue engineering materials. To investigate the biological properties of the gelatin/chitosan-PA IPN hydrogel fibers, L929 fibroblasts were cultured on IPN fibers for up to 5 days. The morphologies of cells growing on the surface of fibers are shown in Fig. 7. The results reveal that almost all the cells were found to grow well, and there is no obvious difference on the quantity per unit area of cells between hydrogel fibers after incubating for 5 days.

In the SEM images of Fig. 8, the cells are observed to penetrate into the hydrogel fibers, and secrete a great deal of extracellular matrices. The results demonstrated that the hydrogel fibers prepared by bienzymatic method can support cell adhesion and proliferation. Thus, the gelatin/chitosan-PA IPN hydrogel fibers not only have excellent biocompatibility, but also possess good biomedical value.

Knittability of IPN Hydrogel Fibers

Hydrogel fibers withstand machine or even hand woven process is a key factor as tissue engineering scaffolds. A continuous filament of gelatin/chitosan-PA IPN hydrogel fibers was cut into staple fibers (15 cm) to test the knittability of IPN hydrogel fibers. As show in Fig. 9, braids of gelatin/chitosan-PA IPN fibers (G15C1.5) were woven by hand with three or more threads. Apparently, the mechanical strength of IPN fiber is enough to satisfy the handling required for post knitting.

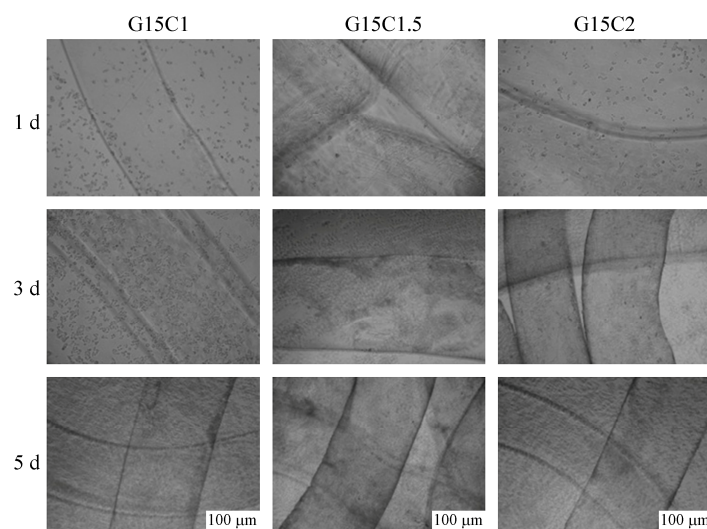


Fig. 7 Optical images of L929 cells cultured on G15C1, G15C1.5 and G15C2 hydrogel fibers with a density of 1×10^5 cells·cm⁻² at 1, 3, and 5 day post-seeding

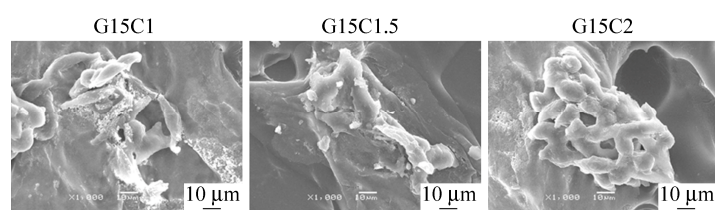


Fig. 8 SEM images of L929 mouse fibroblasts inoculated on gelatin/chitosan-PA IPN hydrogel fibers after 5 days post-seeding

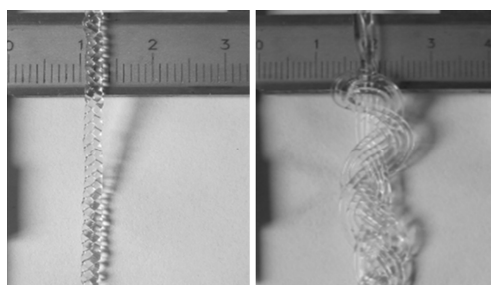


Fig. 9 Photograph of hand-woven braid made from G15C1.5 gelatin/chitosan-PA IPN fibers

CONCLUSIONS

A series of gelatin/chitosan hydrogel fibers with interpenetrating network structure (IPN) were prepared successfully by bienzymatic crosslinking approach without using any toxic chemicals or extraneous cross-linking agents. Owing to the specific IPN structure, the gelatin/chitosan hydrogel fibers showed improved mechanical strength. In addition, the hydrogel fibers have tunable swelling ratio and degradability by adjusting compositions. Furthermore, the preliminary biological evaluation indicated that the IPN hydrogel fibers can support cell adhesion and proliferation effectively. The success in obtaining hand woven braid demonstrated their knittability. Taken together, the bienzymatic crosslinked gelatin/chitosan-PA IPN hydrogel fibers are expected to have potential in the development of tissue engineering scaffolds.

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