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Enzymatic and Ionic Crosslinked Gelatin/K-Carrageenan IPN Hydrogels as Potential Biomaterials

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ABSTRACT: Hydrogels of a natural origin have attracted considerable attention in the field of tissue engineering due to their resemblance to ECM, defined degradability and compatibility with biological systems. In this study, we introduced carrageenan into a gelatin network, creating IPN hydrogels through biological methods of enzymatic and ionic crosslinking. Their gelation processes were monitored and confirmed by rheology analysis. The combination of biochemical and physical crosslinking processes enables the formation of biohydrogels with tunable mechanical properties, swelling ratios and degradation behaviors while maintaining the biocompatibilities of natural materials. The mechanical strength increased with an increase in carrageenan content while swelling ratio and degradability decreased correspondingly. In addition, the IPN hydrogels were shown to support adhesion and proliferation of L929 cell line. All the results highlighted the use of biological crosslinked gelatin-carrageenan IPN hydrogels in the context of tissue engineering. © 2014 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2014**, *131*, 10.1002/app.40975.

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INTRODUCTION

An ideal tissue-engineered polymeric scaffold should be able to provide suitable hydrophilicity, viscoelasticity and biocompatibility to support cellular proliferation and other metabolic activities. Hydrogels exhibit high permeability of oxygen, nutrients and other soluble factors[1] essential for sustaining cellular behaviors.[2,3] Thus, hydrogels have been used as biomaterials in tissue engineering for many years.

Hydrogels can be created by either physical or chemical crosslinking methods. Physical crosslinking, for example ionic crosslinking, are formed due to the electrostatic interactions between charged polymer chains.[4] However, the uncontrollable exchange of ions in biological conditions limits their applications in the biomedical area. On the other hand, the hydrogels synthesized via chemical crosslinking approaches are more stable and possess higher mechanical strength. However, most photo-initiators, thermo-initiators and chemical crosslinkers are cytotoxic (e.g. glutaraldehyde, carbodiimide, and diphenylphosphoryl azide), thereby existing a risk of exposing cells to the residues of toxic chemicals. Hence, biocompatible methods for creating mechanical stable hydrogels are highly sought after in the tissue engineering field.

In terms of the constituting polymers, more interest has been drawn towards natural polymers which are nontoxic and biologically compatible. This provides an advantage over synthetic polymers, ceramic and metallic materials. Among natural polymers, gellan gum,[5,6] alginate,[7] gelatin,[8,9] hyaluronic acid,[10,11] chitosan,[12] and chondroitin sulphate[13] are widely used either alone or combined to achieve unique properties for biomedical and tissue engineering applications.

Carrageenan, a naturally occurring polymer extracted from seaweed, is often used as an inexpensive material in the field of tissue engineering.[14,15] Their primary structure is based on alternating copolymers of 1,3-linked-D-galactose and 1,4-linked R-D-galactose.[16] According to varying degrees of sulfation, there are three main varieties of carrageenan, named as type kappa (κ), iota (γ), and lambda (λ).[17] The aqueous solutions of κ- and γ- carrageenans can form thermo reversible gels upon cooling. Upon a decrease in temperature, random carrageenan molecules undergo a coil-to-double-helix transition, which does not provide a stable network due to the half-ester sulfate moieties that repulse each other by electrostatic interaction.[18,19] The introduction of monovalent cations, such as K^+ ions, could suppress the electrostatic repulsion by binding to the surface of the helices, balancing the negative charges of the half-ester sulfate groups and thereby allowing for a gelation.[20]

Gelatin, another well-known naturally occurring polymer, is a protein obtained from the acidic or alkaline denaturation of collagen.[21] Apart from the traditional crosslinking of

Table I. Composition of the Biopolymer Hydrogels in the Rheological Test

Sample	Gelatin (mg/mL)	KC (mg/mL)	mTG (U/mL)	K^+ (m*M*)
Gel 1	100	10	0	0
Gel 2	100	10	10	0
Gel 3	100	10	0	50
Gel 4	100	10	10	50
Gel 5	100	7	10	50
Gel 6	100	13	10	50

glutaraldehyde, gelatin networks can be formed upon interaction with microbial transglutaminase (mTG).[22,23] The aqueous solution of enzyme catalyzes the formation of isopeptidic bond *N*-ε-(γ-glutamyl)-lysine between two gelatin molecules.[24] Due to the mild gelation condition and excellent cytocompatibility, the mTG catalyzed crosslinking, an interesting alternative to traditional crosslink approaches, has attracted much attention in the past several years.[25,26]

The purpose of this study is to create an IPN scaffold using natural polymers via a combination of enzymatic crosslinking and ionic crosslinking for tissue engineering applications. The natural polymers, carrageenan and gelatin, were selected for the fabrication of IPN hydrogel. By changing the density of one of the networks, adequate control over the swelling properties, degradation characteristic and mechanical properties of the hydrogels can be achieved for different requirements in bioengineering area.

To the best of our knowledge, this is the first study reporting the nontoxic crosslinking of gelatin and κ–carrageenan (KC) with highly versatile physico-chemical properties to achieve different requirements for materials in bioengineering. The IPN structure bestows hydrogels with controllable tensile moduli, swelling ratios and degradability. The rheological and mechanical properties of the IPN hydrogel were monitored in comparison with single network and corresponding semi-IPN hydrogel. The cytocompatibility of the biohydrogels was evaluated by an MTT assay and *in vitro* culture. The results demonstrate that the gelatin-carrageenan IPN gels are an exceptional material for cell culture in tissue engineering areas.

MATERIALS AND METHODS

Materials

κ-Carrageenan (KC) was obtained from Qingdao Ocean Seaweed Industry Co., Ltd. (Shandong, China). Gelatin (Type A), KCl, dimethyl sulfoxide (DMSO) and papain (6000 U/mg) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Microbial transglutaminase (mTG, 1000 U/g) was a gift from Yiming biological products Co. Ltd (Jiangsu, China). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin, l-glutamine, trypsin and 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Invitrogen Co. (Carlsbad, CA). L929 mouse fibroblast cells were purchased from Shanghai Institute of Biochemistry and Cell Biology (China). 0.01*M* phosphate buffered saline (PBS) solution was made by dissolving 7.9 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 1.8 g K_2HPO_4 in 1 L distilled water (pH = 7.4). The chemicals in PBS were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). L929 cells were cultured in complete growth medium prepared with DMEM supplemented with 10% FBS, 1 m*M* L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a 5% CO_2 atmosphere.

Rheological Testing

κ-Carrageenan (KC) and gelatin powder were dispersed by stirring at 60°C for 3 h for complete dissolution. The polymer solutions were then mixed according to the concentrations shown in Table I and kept at 60°C before each rheological measurement to prevent gelation. The mTG stock solution or KCl solution was added to the polymer mixture right before the viscoelastic measurements.

To determine the change of storage modulus (*G'*) and loss modulus (*G"*) in gelation, the rheological measurements were performed using a Rheometer (HAAKE RS600) equipped with a parallel plate geometry of 60 mm diameter, with a solvent cover from HAAKE in order to avoid evaporation. The preheated sample solution was poured directly onto the plate of the instrument without bubbles. Then, an oscillatory logarithmic sweep was started immediately at a frequency of 1 Hz and a strain of 1%. Time dependence of *G'* and *G"* was observed at 55°C for 30 min for mTG enzymatic catalyzed gelation. After attaining the plateau values of both moduli, the temperature dependence of *G'* and *G"* was observed by cooling the system from 55°C to 10°C and then reheating to 55°C, at a rate of 1°C/min.

Preparation and Crosslinking of Hydrogels

The schematic of the crosslinking of gelatin/κ-carrageenan (KC) gels is presented in Figure 1, which highlights the proposed crosslinking mechanisms employed in this study. Briefly, crosslinked gelatin network by enzymatic crosslinking procedure was developed followed by physical crosslinked κ-carrageenan in the presence of K^+ ions. By using the combination of the physical and chemical crosslinking mechanism, the gelatin/κ-carrageenan gel was formed and termed as IPN hydrogel, while the corresponding hydrogel without K^+ treatment was named as semi-IPN hydrogel.

For gelatin/κ-carrageenan IPN hydrogels, corresponding solutions of gelatin and carrageenan as shown in Table II were made by dissolving the polymers separately in distilled water at 60°C to 70°C for 2 h till the solutions become clear followed by mixing. mTG from the stock solution (100 U/mL) were then added to the above mixture with a final concentration of 10 U/mL, followed by thorough stirring. The mixture solution was then immediately poured onto a membrane-shape glass mold with 1.5 mm in thickness and incubated at 55°C for 30 min for enzymatic crosslinking before they were taken out of the mold. The resultant gels were subsequently incubated in a 50 m*M* potassium chloride solution at 4°C for another 1 h for ionically crosslinking. As a control, we have also synthesized gelatin hydrogels and gelatin/κ-carrageenan semi-IPN hydrogels,

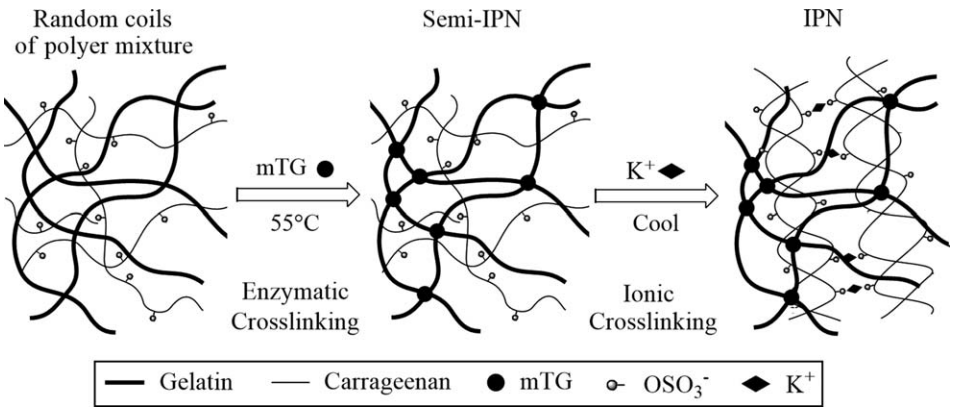

Figure 1. The schematic of the crosslinking of gelatin/κ-carrageenan gels by using a combination of enzymatic and ionic crosslinking methods.

corresponding to polymer solutions shown in Figure 1. Specifically, gelatin and semi-IPN hydrogels were only enzymatically crosslinked as described above without ionic crosslinking. The resultant gelatin, semi-IPN and IPN hydrogel sheets were punched into discs of 4.5 cm^2 and washed in distilled water for 3 h with three changes of water for further swelling, degradation and cytocompatibility tests.

Mechanical Test

To assess the effect of the IPN network and polymer concentration on the mechanical properties of the synthesized hydrogels, tensile tests were performed on an Instron4466 (Series IX) mechanical tester. Gelatin and IPN hydrogel membranes were cut into a dumb bell-shaped gels with 1 mm thickness, 8 mm width and 20 mm length and tested in tensile mode at a rate of 10 mm per minute until failure of the hydrogel.

Swelling Test

To assess the swelling properties, hydrogels ($n = 3$) were allowed to reach equilibrium in the 0.01*M* PBS solution for 24 h and weighed after wiping off excess liquid with a KimWipe paper from the samples surface to determine the wet weight (W_{wet}). Then the samples were lyophilized and weighed to obtain their dry weight (W_{dry}). The swelling ratio was defined as the mass ratio of the wet to the dry weight, as described in the following equation:

$$\text{Swelling ratio} = W_{wet} / W_{dry}$$

Table II. Different Composition of Gelatin and κ-Carrageenan (KC) Hydrogels in Study

Sample	Gelatin (mg/mL)	KC (mg/mL)	mTG (U/mL)	K^+ (0.05*M*)
Single network				
Gelatin	150	0	10	
Semi-IPN (w/o K^+ treatment)				
G-KC1	150	7	10	
G-KC2	150	10	10	
G-KC3	150	13	10	
IPN (w/K^+ treatment)				
G-KC1	150	7	10	Immerse
G-KC2	150	10	10	Immerse
G-KC3	150	13	10	Immerse

In Vitro Enzymatic Degradation

Degradation studies were performed to determine the stability of the scaffolds over time. The constructs were lyophilized and weighed to obtain the initial dry weight (W_0). Then, they were placed in 0.01*M* PBS supplemented with papain (90 U/mL), and incubated at 37°C with shaking at 80 rpm. The dry weights (W_t) of the constructs were then measured at specified time intervals. For each hydrogel composition, three constructs were measured over a 16 h period. The extent of degradation was expressed as a percentage calculated using the following equation:

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

In Vitro Cytotoxicity Evaluation (MTT Assay)

The cytotoxicity of the hydrogels was evaluated by an MTT assay using L929 mouse fibroblasts. Hydrogel scaffolds were sterilized by autoclaving at 121°C for 20 min, followed by incubation in the media containing 10% FBS at an extraction ratio of 0.75 cm^2/mL for 24 h at 4°C. L929 cells were seeded at a density of 1×10^4 cells/well on 96-well plates and cultured at 37°C in a humidified incubator with 5% CO_2. After L929 cells reached 80% confluency, the media was replaced by 20 μL of the extract and 180 μL growth medium. As a control, 200 μL of growth medium was used. Both the test and control plates were incubated at 37°C in a humidified incubator with 5% CO_2 for 5 days while media was changed every two days. MTT assay was performed every other day. On the day of the assay, media was removed from the well followed by incubation in 20 μL MTT assay solution (5 mg/mL in PBS) for 4 h at 37°C with 5% CO_2. Subsequently, MTT was removed carefully and 100 μL of DMSO was added to each well to dissolve the formazan crystals. The developed color was measured at 490 nm using a Microplate Reader (model 680, Bio-Rad). The results were calculated as a percentage of the test group to the control group (cultured in DMEM containing 10% FBS), in turn illustrating the biocompatibility of the material. All the results of the cytotoxicity

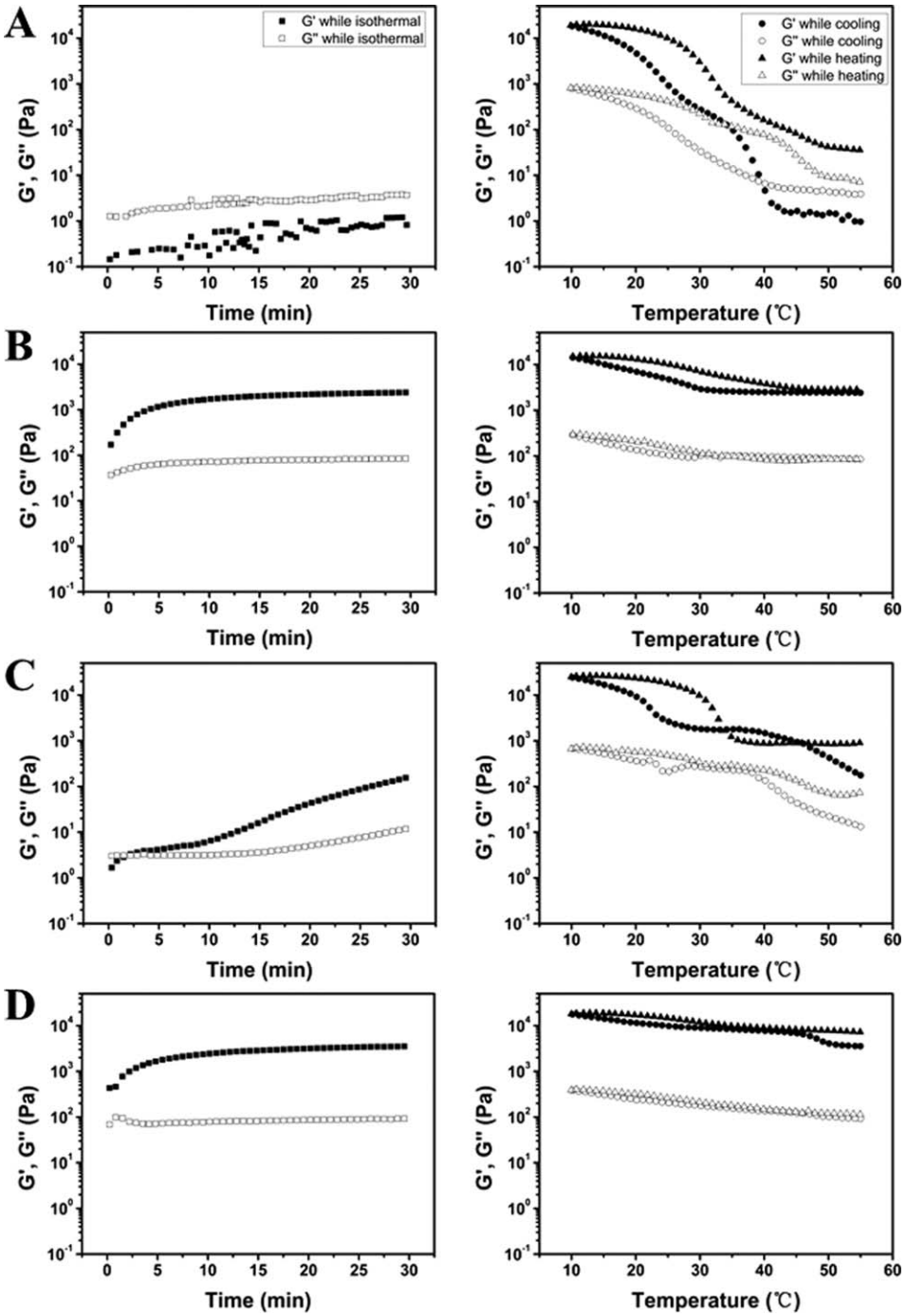

Figure 2. Rheological profiles of gelation process of (A) Gel 1, (B) Gel 2, (C) Gel 3 and (D) Gel 4 at a frequency of 1 Hz and a strain of 1%. The process was performed at 55°C for 30 min, subsequently cooled from 55°C to 10°C then reheated back to 55°C at a rate of 1°C/min. The compositions of Gel 1, Gel 2, Gel 3, and Gel 4 are showed in Table I.

screening tests were obtained by averaging six replicates in each group.

Cell Adhesion and Proliferation on the Hydrogel

The hydrogel scaffolds were sterilized by autoclaving at 121°C for 20 min, followed by incubating them in growth media at 37°C for 2 h. Hydrogels were then placed in 24-well plates and saturated with DMEM containing 10% FBS at 37°C for 2 h. To study the competency of gelatin-carrageenan IPN hydrogels as a scaffold for cell adhesion, L929 cells were used and seeded on the saturated hydrogels at a seeding density of 1×10^4 cells/cm^2 and cultured at 37°C, in a humidified incubator with 5% CO_2 for 7 days, with growth media changed every other day. After 7 days, the cell-seeded hydrogels were removed from growth media and fixed with 2.5% glutaraldehyde solution at room temperature for 2 h and rinsed with PBS and distilled

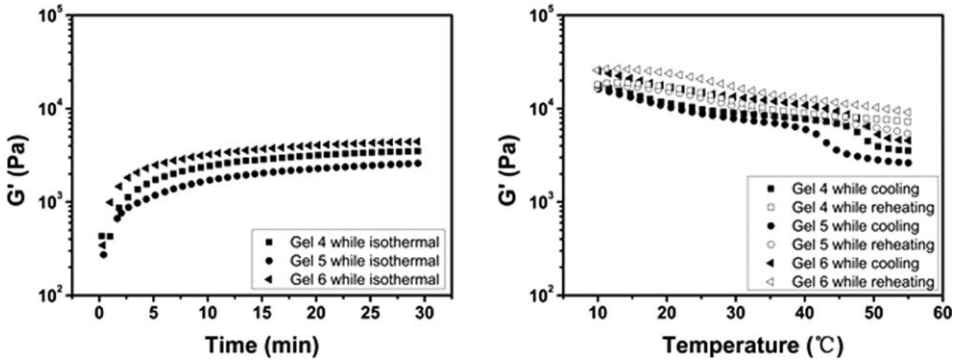

Figure 3. Comparison of the viscoelastic properties of IPN gelation obtained from gelatin/carrageenan with different carrageenan concentration at a frequency of 1 Hz and a strain of 1%. The process was performed at 55°CC for 30 min, subsequently cooled from 55°C to 10°C then reheated back to 55°C at a rate of 1°C/min. The compositions of Gel 4, Gel 5, and Gel 6 are showed in Table I.

water for three times. After fixation, the samples were freeze-dried. Morphologies of the cells on the gels were observed under a scanning electron microscope (SEM) (Mirero AIS2100, Korea) after gold sputter-coating.

RESULTS AND DISCUSSION

Rheological Testing

The rheological experiments were performed to analyze the viscoelastic properties of the gelation of gelatin and carrageenan under different conditions. The storage (G') and loss (G'') moduli parameters were used to monitor the formation of enzyme-catalyzed polymeric networks at 55°C and the physical crosslinking network when cooled to 10°C after the isothermal process. The stabilities of different polymeric networks were analyzed with the change of viscoelastic properties when reheated to 55°C.

Figure 2 shows the representative values of G' and G'' in Gel 1, Gel 2, Gel 3, and Gel 4 in different temperature dependent sweeps. For the isothermal process, a step-like significant increase of G' and G'' was only observed in Gel 2 and Gel 4 [Figure 2(B,D)] by enzymatic crosslinking, indicating the formation of a strong network where the storage moduli were as high as 1.7×10^3 Pa. (The growth of the G' and G'' at initial stage could not be recorded as the moduli increased so dramatically within the first 5 mins due to the rapid enzymatic reactions.) A slight and slow increase of G' was observed in Gel 3 [Figure 2(C)], indicating the formation of a weak network. Different from the enzyme-catalyzed network, this was due to the introduction of potassium cations, which could shield the electrostatic repulsion of the carrageenan molecules and thereby resulting in weak gelation. As a blank control, the G' and G'' of Gel 1 [Figure 2(A)] retained a lower value and liquid-like behavior ($G' < G''$) in the absence of mTG and potassium ions.

Although the G' and G'' of different groups plateaued at different levels after isothermal incubation for 30 min, all of the G' moduli reached an approximate 2×10^4 Pa when cooled from 55°C to 10°C due to the physical crosslinking (ionotropic or thermotropic) of carrageenan molecules. It will be discussed later that the ionotropic network induced by potassium ions is more thermal stable than the thermotropic network in the absence of potassium ions.

The system was then reheated from 10°C to 55°C to determine the effect of mTG and K^+ on the stability of the IPN gels. After reheating, only the G' of Gel 4 [Figure 2(D)] maintained at the same level as that of the low temperature (10^4 Pa), while the G' moduli of the other groups decreased to different extents, indicating the advantage of IPN hydrogels in thermo stability. In the dual network, the first network was crosslinked by enzymatic catalyzed isopeptidic bond while the latter was crosslinked upon cooling under appropriate potassium condition by ionic interactions. In conclusion, the stability of IPN gelation was enhanced markedly in the presence of both mTG and potassium ions.

Under the same condition, IPN groups with different concentrations of carrageenan (Gel 5 and Gel 6) were synthesized to evaluate the effect of IPN structure on the viscoelastic properties of the whole system. As shown in Figure 3, the same trend of G' modulus with Gel 4 was observed in Gel 5 and Gel 6. With the increasing of carrageenan concentration from 7 mg/mL to 13 mg/mL, the G' values with all the different temperature sweeps were increased accordingly. The results indicated the contribution of IPN structure to the network density and the mechanical strength of the hydrogel system.

Mechanical Test

The mechanical properties of gelatin/carrageenan hydrogels were characterized by applying tensile tests on crosslinked hydrogels with different compositions as shown in Table II. As indicated in Figure 4 and Table III, the IPN hydrogels exhibited higher tensile mechanical strength as compared with single network gelatin hydrogel and versatile mechanical properties by varying the carrageenan concentration. The tensile strength of IPN hydrogel G-KC1, G-KC2, and G-KC3 with mTG crosslinking and K^+ treatment were 0.089 MPa~0.11 MPa, and 0.18 MPa, rising up with an increase of carrageenan concentration. On the other hand, the tensile strengths of semi-IPN gels, enzymatic crosslinked without K^+ treatment, were only elevated slightly as compared with the gelatin gel. The results indicated the significant contribution of the IPN structure to the mechanical properties of the hydrogel system.

Swelling Test

The swelling properties of tissue engineered materials significantly affect the mass transport characteristics, such as diffusion

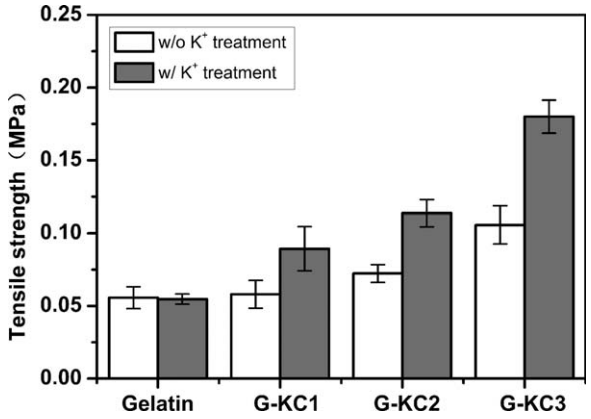

Figure 4. Tensile strength of gelatin, G-KC1, G-KC2, and G-KC3 hydrogels.

of nutrients, oxygen, and waste disposal.[27] As shown in Figures 5, a significant decrease in the mass swelling ratio was observed in the IPN hydrogels G-KC1, G-KC2, and G-KC3, with an increase of ionic crosslinking within the network. However, the swelling ratios of the semi-IPN gel G-KC1, G-KC2, and G-KC3 without K^+ treatment were slightly elevated as compared with the gelatin gels. These observations were dependent on the crosslinking mechanism. The enzymatically crosslinked (chemically bonded) network was flexible and allowed for the extension of the polymeric network through the adsorption of solvent, especially in the presence of uncrosslinked carrageenan which is highly hydrophilic. On the other hand, the ionic bonds between the carrageenan chains by potassium chloride treatment (physical crosslinking) were less flexible; hence the ability of the IPN hydrogels to retain the solvent is reduced by combining both crosslinking mechanisms.

In Vitro Enzymatic Degradation

The degradation characteristics of all hydrogels were examined by incubating samples in 0.01*M* PBS (pH 7.4) at 37°C. The gelatin/carrageenan IPN hydrogels showed a higher degree of degradation as compared with gelatin hydrogels, which showed a 17% degradation after 16 h whereas the IPN gels degraded completely (Figure 6). These results may be due to the instability of ionically crosslinked network. With the gradual proteolytic degradation of gelatin by papain, the carrageenan network might collapse with uncontrollable exchange of monovalent ions with other positive ions from the surrounding PBS solution. Due to the simultaneous dual degradation mechanism, the IPN gels degraded faster than the gelatin gel as the latter is only proteolytically cleaved by papain in this study. On the other hand, with the increase of ionically crosslinking network of carrageenan the degradation rates were slightly decreased in the whole degradation process. These differences may be contributed to the denser network structure beard by higher concentration of carrageenan.

In Vitro Cytotoxicity Evaluation

The soluble materials leached from the hydrogels were evaluated using an MTT assay. Figure 7 shows the cellular metabolic activity obtained using extracts of the IPN hydrogels in this study. The results revealed that the cell viability of experimental

Table III. Tensile Mechanical Properties of Gelatin, G-KC1, G-KC2, and G-KC3

	w/o K^+ treatment			w/ K^+ treatment		
Sample	Stress (MPa)	Strain (%)	Modulus (MPa)	Stress (MPa)	Strain (%)	Modulus (MPa)
Gelatin	0.056	0.41	0.14	0.055	0.47	0.12
G-KC1	0.058	0.36	0.15	0.089	0.51	0.19
G-KC2	0.072	0.48	0.15	0.114	0.55	0.23
G-KC3	0.106	0.55	0.23	0.18	0.62	0.30

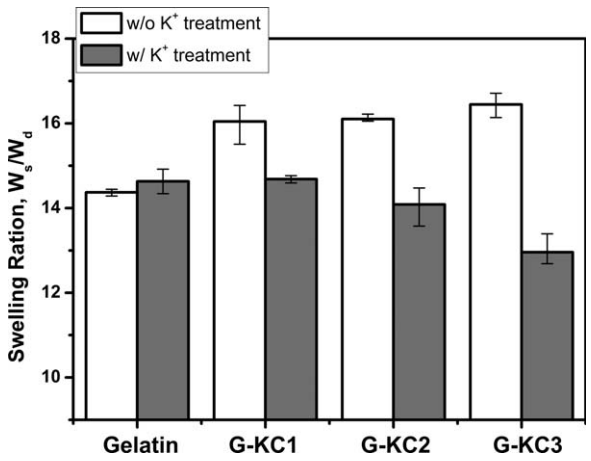

Figure 5. Swelling ratios of gelatin, G-KC1, G-KC2, and G-KC3 in PBS.

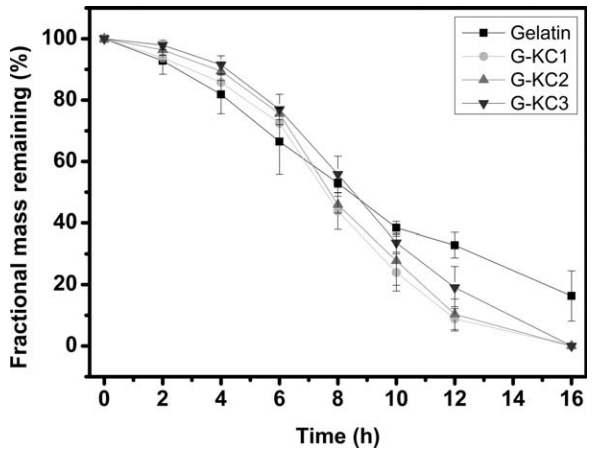

Figure 6. Weight change profiles of gelatin, G-KC1, G-KC2 and G-KC3 hydrogels in PBS containing papain (90 U/mL) at 37°C.

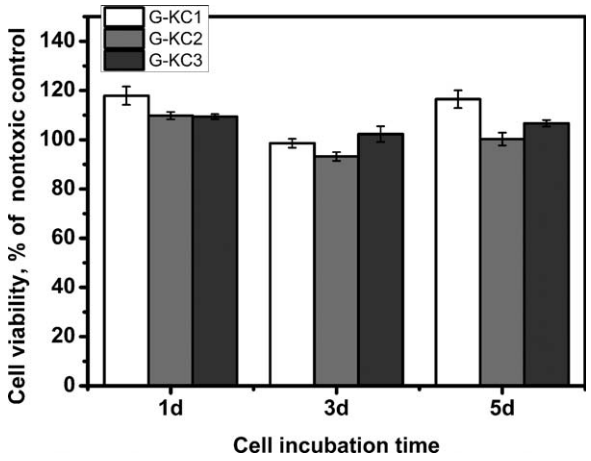

Figure 7. Cytotoxicity of extracts of G-KC1, G-KC2, and G-KC3 in comparison with nontoxic control (growth media only).

groups (cultured in the growth medium supplemented with extract solution of IPN gels) is comparable to the control group (cells with growth medium only), and the experiment groups even supported the metabolic activities of cell line L929 with cell viability higher than 100%. The test demonstrated high cytocompatible levels of the IPN hydrogel system.

Cell Adhesion and Proliferation on the Hydrogel

The morphologies of the L929 cell line seeded on the gelatin and IPN gels were shown in Figure 8. There is no significant difference of cell viability between gelatin and gelatin/carrageenan gel. The SEM [Figure 8(B)] images indicated that the G-KC1, G-KC2, and G-KC supported the L929 cells attaching and proliferating up to 7 days. In conclusion, the gelatin/carrageenan system is extremely comparable to gelatin which is widely used in tissue engineering.

CONCLUSION

In conclusion, the polymeric hydrogel matrix consisting of two natural polymers, gelatin and carrageenan, was fabricated by two different networks using natural enzyme mTG and potassium cations. The combination of physical and chemical cross-linking procedure led to the formation of thermo-stable hydrogels with versatile physical performance. These physical properties, such as mechanical strength and swelling ratio, can be easily tailored by varying the concentration of carrageenan. These biohydrogels also demonstrated a biodegradable nature and excellent cytocompatibility. Cell matrix interaction was also performed with cell line L929, showing excellent cell adhesion and proliferation within the hydrogel system. Taken together, the carrageenan-gelatin matrices with IPN structure prepared by

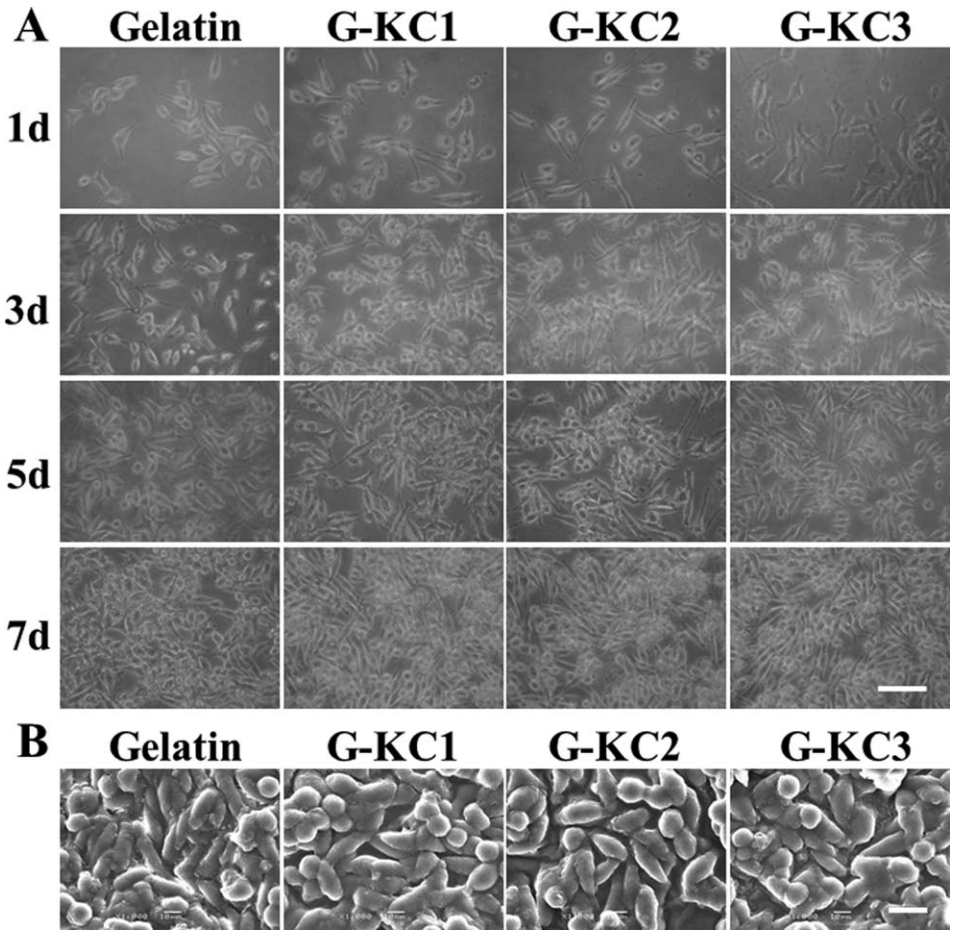

Figure 8. Adhesion and growth of L929 mouse fibroblasts on Gelatin, G-KC1, G-KC2, and G-KC3 hydrogels. (A) Photomicrograph of L929 cells growing on hydrogels at 1, 3, 5, and 7 day post-seeding (scale bar 100μm). (B) SEM images after 7 days post-seeding (scale bar 20 μm).

biocompatible process showed promising results as a potential scaffold for tissue engineering applications.

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