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A green fabrication approach of gelatin/CM-chitosan hybrid hydrogel for wound healing

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ABSTRACT

A series of hydrogels were fabricated from an aqueous solution of gelatin and carboxymethyl chitosan (CM-chitosan) by radiation-induced-crosslinking at ambient temperature. Several physicochemical and biological properties of the hydrogels were investigated to evaluate their potential as wound healing materials. By increasing the CM-chitosan content in the hybrid hydrogels, their swelling ability increased significantly, while the compressive modulus decreased. The miscibility between gelatin and CM-chitosan molecules led to a semi-interpenetrate network after crosslinking. Observed by SEM, scaffolds with a homogeneous interconnected pore structure were obtained after lyophilizing the hydrogels. The enzyme degradation rate of the hydrogels was controlled by adjusting the procedure, which could be matched to the healing rate of the wound. Furthermore, the gelatin/CM-chitosan hydrogels promoted cell attachment and rapid growth of fibroblasts on the material. Due to the high water absorption capacity, a similar compressive modulus with soft tissue, controllable biodegradation, and excellent biocompatibility, the gelatin/CM-chitosan hybrid hydrogels have potential as skin scaffolds and wound healing materials.

1. Introduction

An ideal biomimic scaffold for soft tissue regeneration could be composed of protein and polysaccharide, with a highly interconnected porous structure. Hydrogels have been frequently utilized as scaffolds for soft tissue due to their excellent biocompatibility, biomimic microstructure and mechanical properties (Drury & Mooney, 2003; Jeon et al., 2007; Nguyen & West, 2002).

Natural polymers have similar components with native extracellular matrices (ECM) and are widely used for biomedical applications. Gelatin and chitosan derivatives are among the most frequently used biomaterials due to their biocompatibility. Gelatin, a derivative of collagen, is fabricated by breaking the natural triple-helix structure of collagen to single strand molecules through hydrolysis. Gelatin contains a number of biological functional groups like amino acids and has been clinically used as a wound dressing. Its potential as artificial skin, bone grafts and pharmaceutics has been intensively investigated (Marois et al., 1995). However, gelatin is generally crosslinked by chemical methods, which inevitably use toxic crosslinkers or initiators, and therefore cause a cytotoxicity problem. Besides, gelatin hydrogels have certain drawbacks which are unfavorable for wound healing, i.e. insufficient flexibility and water absorption capacity, they readily cause imflammation and are easily deteriorated by bacteria during the application period.

Chitin and chitosan are regarded as appropriate biomaterials due to their physicochemical and biological properties. However, acetic acid or organic solvents should be applied for material preparation, which would impart certain cytotoxicity to the final product (Shanmugasundaram et al., 2001). Carboxymethyl chitosan (CM-chitosan), a water-soluble derivative of chitosan, has the merits of chitosan and has improved biocompatibility over chitosan (Chen, Wang, Liu, & Park, 2002; Zhu & Fang, 2005). Therefore, CM-chitosan has been extensively utilized in biomedical materials including moisture-retention agents, bactericides, wound dressings, artificial skin, blood anticoagulants and so on (Hirano, 1996; Muzzarelli, 1988; Muzzarelli, Tanfani, Emanuelli, & Mariotti, 1982; Zhang, Guo, Zhou, Yang, & Du, 2000). Furthermore, CM-chitosan was capable of

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stimulating the extracellular lysozyme activity of fibroblasts, promoting the proliferation of normal skin fibroblasts and inhibiting the proliferation of keloid fibroblasts (Chen et al., 2002).

Currently, several crosslinking methods have been employed to prepare hydrogels, including thermal heating, ultraviolet irradiation, and chemical crosslinking. Various crosslinkers such as glutaraldehyde (Liu, Ee, Ke, Hedrick, & Yang, 2009; Welsh & Tirrell, 2000; Zhang, Venugopal, Huang, Lim, & Ramakrishna, 2006), bis(sulfosuccinimidyl) suberate (McMillan, Caran, Apkarian, & Conticello, 1999; McMillan & Conticello, 2000; Mithieux, Rasko, & Weiss, 2004), D,L-glyceraldehyde (Kosmala, Henthorn, & Brannon-Peppas, 2000) and genipin (Bigi, Cojazzi, Panzavolta, Roveri, & Rubini, 2002) have been used to cross-link gelatin, chitosan and elastin in aqueous or organic solutions. Although the treatment by chemical reagents are effective in yielding a high degree of crosslinking, the chemical residuals are highly cytotoxic, which restrict their potential applications in the biomedical field. On the contrary, a radiation crosslinking technique is a green approach to fabricate hydrogels because the entire sample preparation procedure is performed in a pure water system. So, the prepared materials are additive-free and sterilized (Qiu et al., 2007). Plenty of polysaccharide hydrogels (Yoshii et al., 2003; Zhao, Mitomo, Nagasawa, Yoshii, & Kume, 2003; Zhao, Mitomo, & Yoshii, 2008; Zhao et al., 2003; Zhao, Xu, Mitomo, & Yoshii, 2006) and a few gelatin hydrogels are prepared by radiation crosslinking (Cataldo, Ursini, Lilla, & Angelini, 2008; Kojima, Bessho, Furuta, Okuda, & Hara, 2004). Nevertheless, their properties related to biomedical applications are not well investigated.

This work presents the first attempt to prepare a polysaccharide/protein, i.e. gelatin/CM-chitosan, hybrid hydrogel by radiation crosslinking. Physicochemical properties of the hydrogels such as gel fraction, swelling behavior, morphology, and mechanical strength were investigated. Their biological properties, such as in vitro biodegradation behavior, cytotoxicity, cell attachment and proliferation of fibroblasts on the hydrogels, were evaluated. On account of their biomimic composition and a green fabrication procedure, the gelatin/CM-chitosan hybrid hydrogels are expected to have marvelous prospects as scaffolds for soft tissue regeneration or wound dressing.

2. Material and methods

2.1. Materials

Gelatin type A (porcine, bloom 300, from Sigma–Aldrich) and N,O-carboxymethylated chitosan (Mw 54,500, degree of deacetylation 90%, degree of substitution 1.78, from Jinhu Co. Ltd., China) were used to prepare hydrogels. Lysozyme (ultra pure grade, activity > 20,000 U/mg, Amresco 0663) and collagenase type I (activity > 125 U/mg, Sigma C0130) were applied in an enzyme degradation study. The other chemicals were analytical reagents and used as received.

2.2. Preparation of the hydrogels

Gelatin, CM-chitosan powder, and distilled water at different ratios were mixed by a ARE-310 hybrid mixer (Japan Thinky Co. Ltd.) for 10 min to form a homogenous solution. The total polymer concentration was fixed to 15% by weight with the ratios of gelatin to CM-chitosan at 10/0, 8/2, 6/4, 4/6, 2/8 and 0/10, respectively. The solutions were filled into test tubes (inner diameter 10 mm) and subjected for γ-irradiation using a ^{60}Co radiation facility, which was performed at room temperature with a dose rate of 20 Gy min^{-1} at a desired absorbed dose.

2.3. Gel fraction

The hydrogels were cut into cylinders with a diameter of 10 mm and thickness of 10 mm. The hydrogel cylinders were weighed (W_i) and immersed in distilled water for 5 days at 25 °C in order to remove the soluble components. The remaining gel was then dried in a vacuum at 60 °C to constant weight (W_d). The gel fraction was calculated using Eq. (1):

$$\text{gel fraction} = \frac{W_d}{W_i \times a} \times 100\% \tag{1}$$

where W_d is the weight of the dry gel after removing soluble portion; W_i is the initial weight of the sample; "a" denotes the polymer concentration of the hydrogel samples, which is equivalent to the polymer concentration (0.15 in this study). Data from each sample was calculated using triplicate measurement.

2.4. Swelling behavior

2.4.1. Equilibrium swelling in water

The hydrogel cylinders (diameter 10 mm, thickness 10 mm) were immersed in distilled water at 25 °C for 5 days to reach equilibrium. The swollen samples were taken from the water and weighed (W_w). Prior to weighing, excessive water in the samples was gently removed using a filter paper. The samples were then dried at 60 °C until reaching a constant weight (W_d). The equilibrium degree of swelling (EDS) was calculated using Eq. (2). Data from each sample was calculated using triplicate measurement.

$$\text{EDS} = \frac{W_w - W_d}{W_d} \times 100\% \tag{2}$$

2.4.2. Swelling kinetics in PBS

Swelling kinetics of the hydrogels (prepared by 30 kGy irradiation) in phosphate buffer solution (PBS) (0.15 mol L^{-1}, pH = 7.2) was conducted by determining the weight of the swollen hydrogels at 37 °C at various time points. The degree of swelling was calculated using Eq. (3). Data from each sample was calculated using triplicate measurement.

$$\text{Swelling degree} = \frac{W_P - W_i \times a}{W_i \times a} \times 100\% \tag{3}$$

where W_p is the weight of hydrogels swollen in PBS, W_i is the initial weight of hydrogel samples, and "a" is the polymer concentration of the hydrogel samples, which is equivalent to the polymer concentration (0.15 in this study).

2.5. Mechanical properties

The mechanical properties of the hydrogels were determined by conducting compression tests using a texture analyzer (TMS-PRO, Food Technology Corporation, USA) equipped with a 1 kN load cell. The compression tests were performed on the hydrogel cylinders (10 mm in diameter and 10 mm in thickness, prepared by 30 kGy irradiation) at a constant strain rate of 1 mm/s. Compressive modules of the hybrid hydrogels were determined by the average slope of the stress–strain curve over the strain range of 0–5%. Data from each sample was calculated using triplicate measurement.

2.6. FT-IR analysis

The hydrogel samples (prepared by 30 kGy irradiation) were lyophilized after removing the soluble portion. Fourier transform infra-red (FT-IR) analysis was performed using a Nicolet Magna-IR 750, with a Nicolet NicPlan IR microscope attachment (resolution 2 cm^{-1}, scan 64 times) and a MCT/A detector with a range of 500–4000 cm^{-1}.

2.7. Morphology

Equilibrium swollen hydrogel samples (prepared by 30 kGy irradiation) were lyophilized. The cross-sections of the lyophilized samples were visualized using a scanning electron microscopy (SEM) (Quanta 200) with an accelerating voltage of 15 kV.

2.8. In vitro enzyme degradation

In vitro degradability of the hydrogels in lysozyme and collagenase was investigated. Enzyme solutions, with a concentration of 0.4 mg/ml for lysozyme and 0.1 mg/ml for collagenase, were prepared by dissolving the enzyme powder in PBS (pH = 7.4) at room temperature. The hydrogels (prepared by 30 kGy irradiation) were cut into cylinders with a thickness of ca. 5 mm. After weighing (W_a), the samples were incubated in an enzyme solution at 37 °C. At pre-determined time intervals, the remaining hydrogels were separated from the enzyme solution and dried in a vacuum at 50 °C to constant weight (W_b). The weight loss caused by the enzyme degradation was calculated using Eq. (4). Data from each sample was calculated using triplicate measurement.

$$\text{weight loss} = \frac{(W_a \times a - W_b)}{W_a \times a} \times 100\% \tag{4}$$

2.9. In vitro cell culture study

2.9.1. Cell culture

L929 mouse fibroblasts and NIH/3T3 fibroblast cells (American Type Culture Collection, ATCC) were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The media was replaced every other day. After reaching 80% confluency, the cells were detached by trypsin-EDTA and viable cells were counted using a trypan blue assay. Cells were cultivated at 37 °C and 5% CO_2.

2.9.2. Cytotoxicity of the hydrogel extract

Cytotoxicity of the hydrogels was evaluated by MTT assay under the guideline of international standard ISO 10993-5 (Kasahara et al., 2003; Loh, Sng, & Li, 2008). To prepare the extraction media in a standard culturing condition, the sterilized hydrogels (prepared by 30 kGy irradiation) were incubated for 72 h in a DMEM serum free medium with a surface area/extraction medium ratio of 0.75 cm^2/ml. Following, the mixture of DMEM and the hydrogel was centrifuged (8000 r/min for 10 min at 25 °C) and the supernatant fluid was withdrawn as the extraction medium. Prior to cytotoxicity testing, the extraction was supplemented with 10% FBS and then diluted with DMEM culture medium to obtain extraction medium samples with concentration of 20%, 60%, and 100% (relative to the original extracts). DMEM medium was used as a negative control, and 10% dimethylsulfoxide (DMSO) dissolved in the DMEM medium was used as a positive controls. L929 cells were incubated in a 96-well cell culture plate at a density of 3×10^3 cells per well for 24 h. The culture medium was then removed and replaced with the prepared extraction medium and incubated for 1, 2 and 3 days, respectively. At each time, a 10 μl MTT (5 mg/ml in PBS) was pipetted into each well and incubated for 4 h at 37 °C, then 100 μl SDS (Sodium Dodecyl Sulfate) solution (10%SDS in 0.01 M HCl) was added to each well overnight in a standard culturing condition. The absorbance was measured at 570 nm using a microplate reader (Bio-RAD680). The relative growth rate (RGR) was defined in Eq. (5). Data was calculated from the average of six measurements of two parallel experiments, i.e. triplicate for each experiment.

$$\text{RGR} = \frac{\text{absorbance of sample}}{\text{absorbance of negative control}} \times 100\% \tag{5}$$

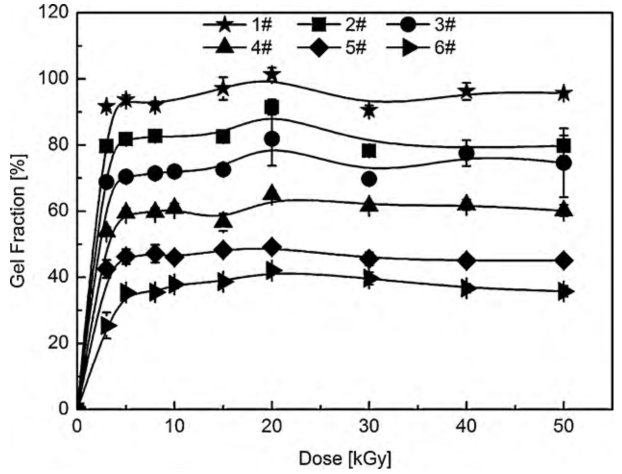

Fig. 1. Gel fraction of gelatin/CM-chitosan hydrogels prepared at different absorbed dose. The weight ratios of gelatin to CM-chitosan = 10:0(1#), 8:2(2#), 6:4(3#), 4:6(4#), 2:8(5#), 0:10(6#).

2.9.3. Cell proliferation on hydrogels

The ability of NIH 3T3 fibroblast cells to proliferate on or into the three-dimensional structure was further evaluated. The hydrogel discs (10 mm in diameter and 2 mm in thickness, prepared and sterilized at the same time by 30 kGy irradiation) were placed in 24-well TCP. 2 mL DMEM serum free media was added in each well for swelling of the hydrogels. After incubated at 37 °C for 48 h, the media was replaced with DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. NIH/3T3 fibroblast cells were then seeded on the hydrogels at an initial density of 5×10^4 cells/well and incubated for 1 day. Thereafter, the incubation media was changed every 2 days. TCP wells without hydrogel samples were used as controls. After culturing for 1, 3 and 5 days in a standard culturing condition, the RGR of cells proliferated on different hydrogel samples were evaluated by MTT assay, in the same manner as used in the cytotoxicity study. All data were analyzed from six measurements of two parallel experiments, i.e. triplicate for each experiment.

2.10. Statistical analysis

All data were expressed as mean ± standard deviations (SD). Statistical significance of differences between means was determined by one-way analysis of variance (ANOVA). *P*-values less than 0.05 denote statistical significance.

3. Result and discussion

3.1. Gel fraction

The gel fraction of gelatin/CM-chitosan hydrogels versus the absorbed dose is shown in Fig. 1. In the groups of all compositions, the gel fraction ascended rapidly initially and then leveled off over the dose of ca. 10 kGy, while it declined slightly at a higher absorbed dose. This phenomenon is universal for the formation of hydrogels via radiation crosslinking. Initially, the amount of free radicals generated by γ-irradiation was not high enough for recombination to form a physically stable hydrogel. However, when the absorbed dose reached a critical absorbed dose (gelation dose), the intermolecular recombination of free radicals readily occurred, and finally led to the formation of a three-dimensional network. Further increase of the absorbed dose would cause increased crosslinking density, while the fraction of the insoluble portion did not change significantly in a certain dose range. At a sufficient high absorbed

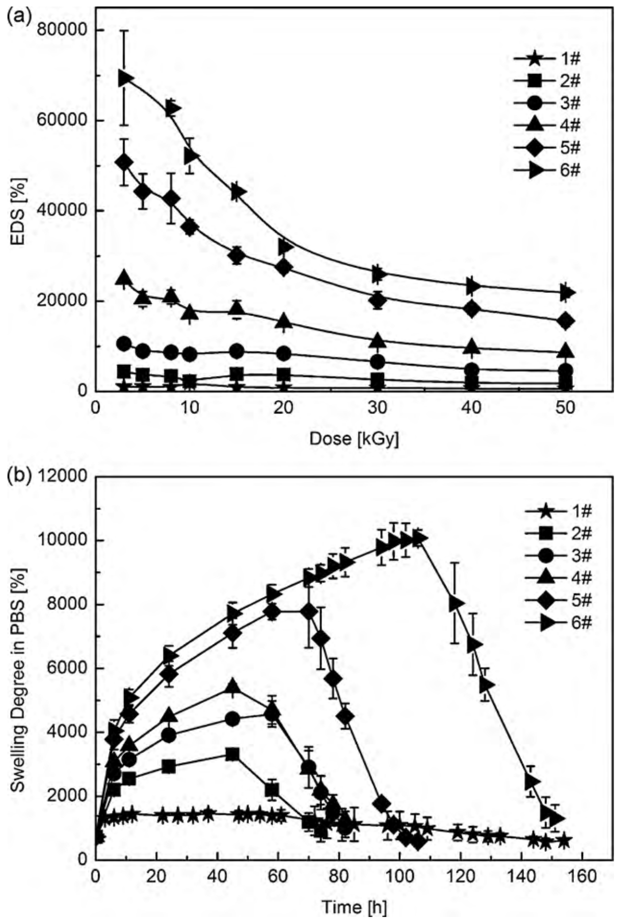

Fig. 2. Equilibrium degree of swelling (EDS) of gelatin/CM-chitosan hydrogels prepared at different absorbed dose (a), and swelling isotherm of gelatin/CM-chitosan hydrogels prepared at 30 kGy in phosphate swelling solution (PBS). The weight ratios of gelatin to CM-chitosan = 10:0(1#), 8:2(2#), 6:4(3#), 4:6(4#), 2:8(5#), 0:10(6#).

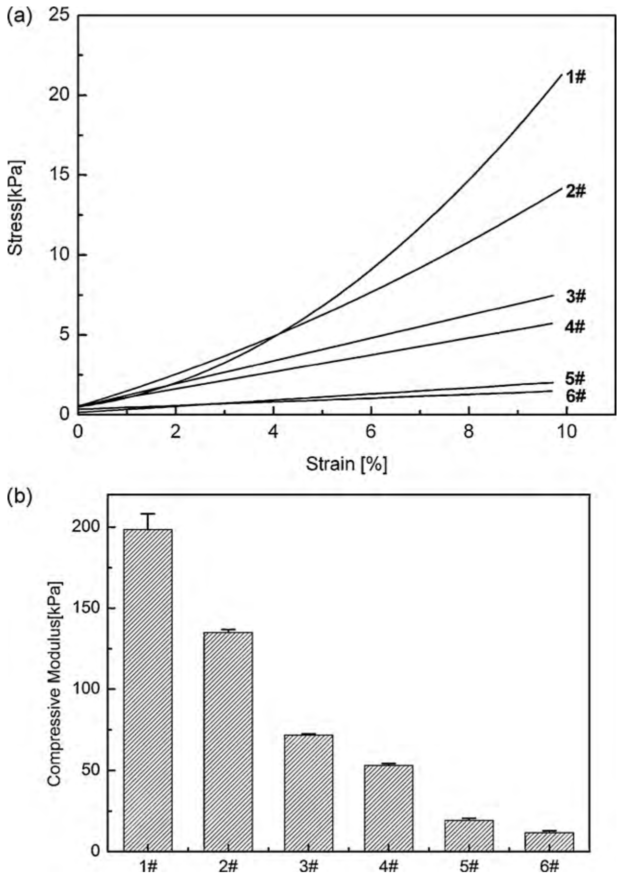

Fig. 3. Stress–strain curve (a) and compressive modulus (b) of gelatin/CM-chitosan hydrogels. The hydrogels were prepared at 30 kGy and the weight ratios of gelatin to CM-chitosan were 10:0(1#), 8:2(2#), 6:4(3#), 4:6(4#), 2:8(5#) and 0:10(6#), respectively.

dose, the intramolecular free radical recombination tended to dominate over the intermolecular radical recombination, which would terminate the crosslinking and begin to degrade the network. Therefore, the gel fraction slightly decreased (Qiu et al., 2007; Zhao et al., 2006).

Polysaccharides are degraded by ionization irradiation in solid state and low concentration solutions. However, a large variety of polysaccharide hydrogels are prepared in concentrated solutions (named as “paste-like status”) by radiation crosslinking (Yoshii et al., 2003; Zhao, Mitomo, Nagasawa et al., 2003; Zhao et al., 2008; Zhao, Mitomo, Zhai et al., 2003; Zhao et al., 2006). Under γ-irradiation, CM-chitosan crosslinked and degraded concurrently. Therefore, although a physically stable hydrogel can be formed, maximum gel fraction of the pure CM-chitosan hydrogel was rather low, i.e. 40%. On the contrary, the radiation crosslinking efficiency of gelatin was much higher than that of CM-chitosan. For instance, the gel fraction of pure gelatin hydrogel was 92% at a low gelation dose (3 kGy). Consequently, the gel fraction of gelatin/CM-chitosan hybrid hydrogel decreased with the increasing content of CM-chitosan.

3.2. Swelling behavior

3.2.1. Equilibrium swelling in water

The water retention ability of gelatin/CM-chitosan hybrid hydrogels, which is an important feature of wound dressing, was investigated. As shown in Fig. 2(a), the EDS of pure CM-chitosan hydrogel in water was approximately 100 times higher than that of the pure gelatin gel, and EDS of the gelatin/CM-chitosan hydrogels prepared by the same absorbed dose increased as the ratio of gelatin/CM-chitosan decreased.

With the increase of the absorbed dose, EDS of the hydrogels decreased and tended to reach a constant when the absorbed dose exceeds 30 kGy. As described in Section 3.1, the gel fraction reached maximum of approximately 10 kGy. However, the crosslinking density of the hydrogels increased continuously until a higher dose, resulting in the continuous reduction of EDS. The crosslinking density also depends on the composition of the hydrogel, the swelling degree of the CM-chitosan/CM-cellulose hydrogels prepared at the same dose decreased with the increasing of gelatin/CM-chitosan ratio (Hiroki, Tran, Nagasawa, Yagi, & Tamada, 2009). In this work, the decrement extent of pure CM-chitosan and gelatin/CM-chitosan = 2/8 was also more significant than other groups because the radiation degradation of CM-chitosan occurred at the same time with radiation crosslinking, leading to the comparatively weak network.

3.2.2. Swelling isotherm in PBS

Swelling behavior of the hydrogels in PBS was investigated to evaluate their capacity to absorb wound exudation fluid, and to simulate their degradation in the presence of body fluid. Fig. 2(b) shows the swelling isotherm of the hydrogel incubated at 37 °C in PBS. It is clear that all of the hydrogel samples degraded after a swelling period. The degree of swelling and swelling kinetics significantly correlated to the hydrogel composition. Pure gelatin hydrogel was slightly swelled in PBS. On the contrary, pure CM-

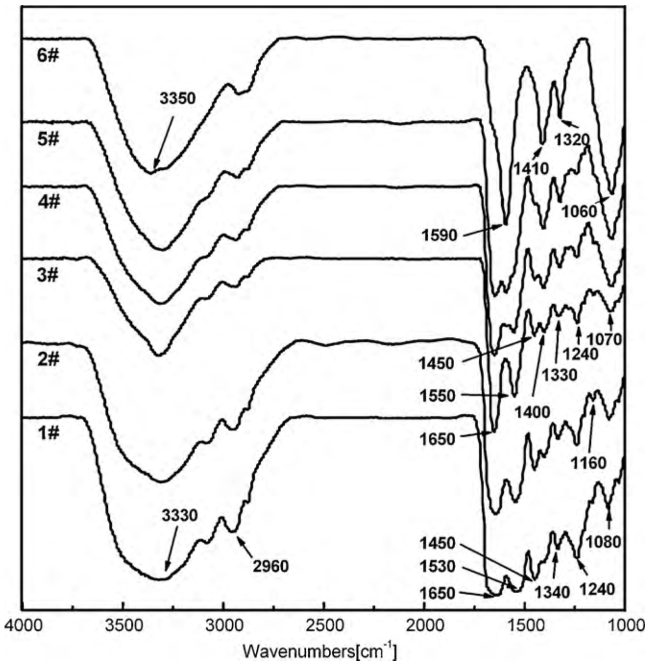

Fig. 4. FT-IR spectra of lyophilize gelatin/CM-chitosan hydrogels. The hydrogels were prepared at 30 kGy and the weight ratios of gelatin to CM-chitosan were 10:0(1#), 8:2(2#), 6:4(3#), 4:6(4#), 2:8(5#) and 0:10(6#), respectively.

chitosan hydrogels swelled remarkably in PBS. The maximum degree of swelling of pure CM-chitosan hydrogel was approximately 9 times higher than that of the pure gelatin gel. The maximum degree of swelling of the pure gelatin hydrogel was reached after 60 h of incubation, while the swelling of samples 2#–6# were delayed with the ratio of gelatin/CM-chitosan.

In the presence of PBS (0.15 mol L^{-1}, pH = 7.2), the hydrogels swelled and degraded at the same time. Initially, PBS diffused into the hydrogels before degradation, leading to the hydrogel swelling. At pH 7.2, the amine group and carboxyl group were not ionized so that they interact with each other via hydrogen bond. The ions in PBS will interrupted the hydrogen bond, resulting in a lower swelling degree of hydrogel in PBS than in pure water. However, after the hydrogels reached the maximum degree of swelling, the rate of PBS diffusion into the bulk of the hydrogels decreased and hydrolysis in the interior of the hydrogel domain, leading to the collapse of the hydrogel. Polysaccharides are readily to be degraded by oxidation and beta-elimination surrounding by acid, alkaline or enzyme. The large divalent ions in PBS such as Ca^{2+} will serve as Lewis acids and accelerated the hydrolysis of CM-chitosan, therefore, lead to the collapse of gelatin/CM-chitosan hydrogels. Surely, hydrogels with higher ratio of gelatin/CM-chitosan will degraded faster than the others.

Most importantly, the incorporation of CM-chitosan clearly improved the swelling ability of the gelatin, which is beneficial to applications with a large amount of exudation fluid. Furthermore, the partially degraded CM-chitosan will be extracted to stimulate the extracellular lysozyme activity of fibroblasts, to promote the proliferation of normal skin fibroblast, and to inhibit the proliferation of keloid fibroblasts, resulting in the acceleration of wound healing and tissue regeneration (Chen et al., 2002).

3.3. Mechanical properties

Mechanical properties, especially compressive strength, are particularly crucial for scaffolds used in tissue engineering. Fig. 3 shows the stress–strain curves and compressive modulus of the gelatin/CM-chitosan hybrid hydrogels prepared at 30 kGy irradiation. The pure gelatin and CM-chitosan hydrogel yielded the highest and lowest stress, while the stress of the hybrid hydrogels decreased as the ratio of gelatin/CM-chitosan decreased. The com-

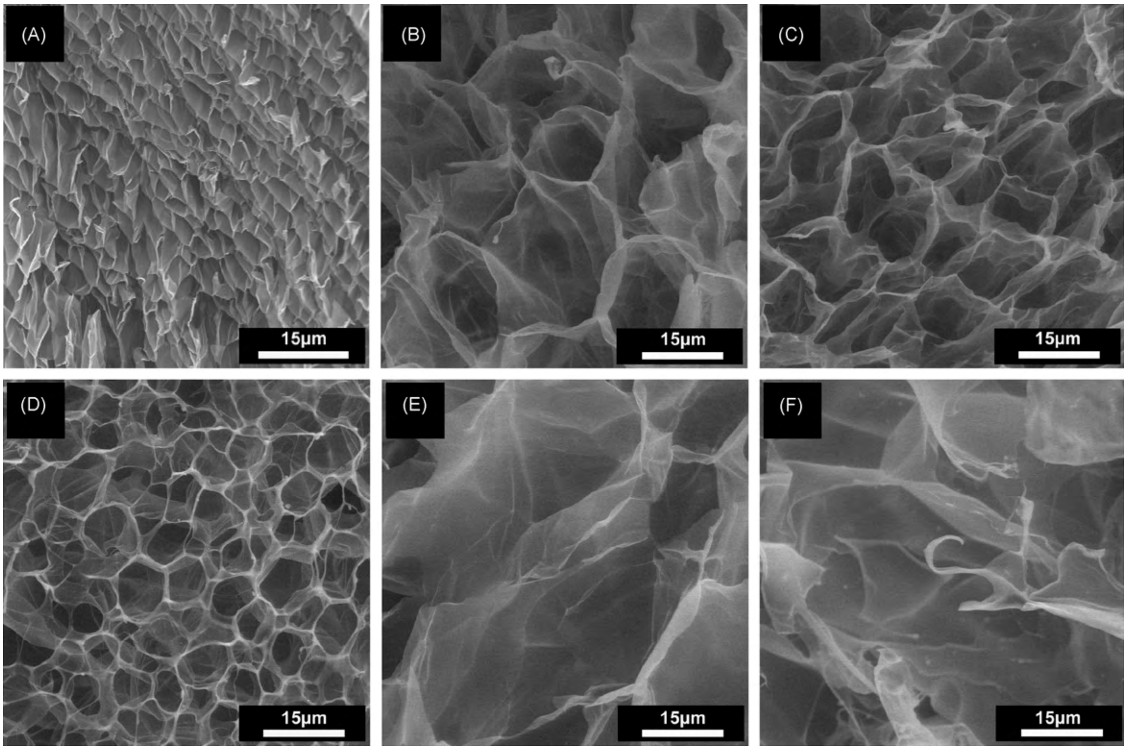

Fig. 5. The scanning electron micrographs of the lyophilized gelatin/CM-chitosan hydrogels. The hydrogels were prepared at 30 kGy and the weight ratios of gelatin to CM-chitosan were 10:0(1#), 8:2(2#), 6:4(3#), 4:6(4#), 2:8(5#) and 0:10(6#), respectively.

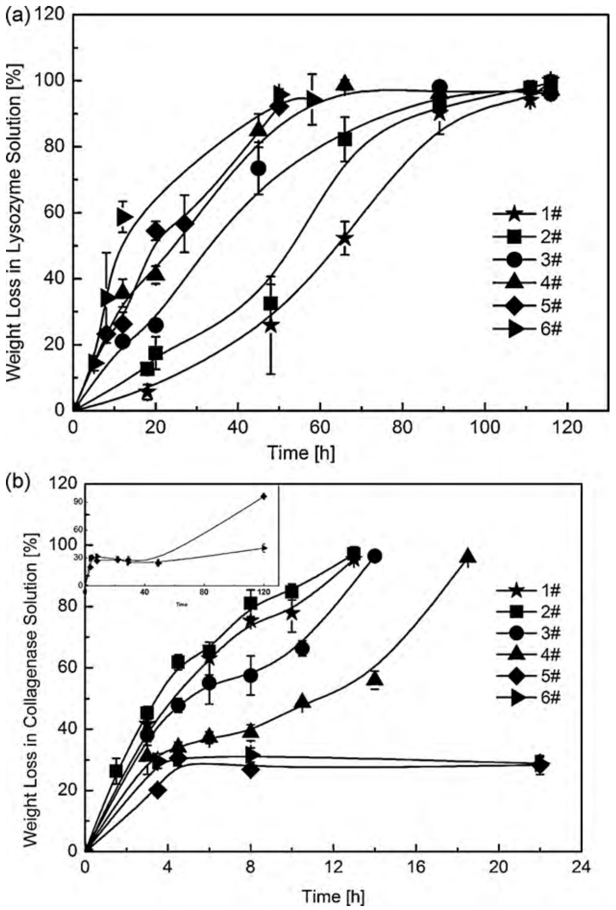

Fig. 6. The weight loss for the gelatin/CM-chitosan hydrogels incubated in 0.4 mg/ml lysozyme solution (a) and 0.1 mg/ml collagense solution (b). The hydrogels were prepared at 30 kGy and the weight ratios of gelatin to CM-chitosan were 10:0(1#), 8:2(2#), 6:4(3#), 4:6(4#), 2:8(5#) and 0:10(6#), respectively.

pressive modulus significantly decreased from 198 kPa (gelatin) to 11.6 kPa (CM-chitosan) with the increase of the CM-chitosan content. The gelatin/CM-chitosan hybrid hydrogels exhibited favorable mechanical properties for soft tissue repair. Particularly, the compressive modulus of 3#–5 hydrogel samples were similar to those of the skin in various positions in humans, demonstrating their superior biomimic properties compared with pure gelatin and CM-chitosan hydrogel (Dawes-Higgs, Swain, Higgs, Appleyard, & Kossard, 2004; Lu, Yu, Huang, Huang, & Zheng, 2009).

3.4. *FT-IR analysis*

The FT-IR spectra of the cross-linked hydrogles after extracting soluble parts are shown in Fig. 4. The absorption bands at 3300, 1590, 1410 and 1060 cm^{-1} in the spectrum of CM-chitosan were assigned to the O–H stretching vibration, N–H stretching vibration of amide (II), the symmetric stretching vibrations of COO^{-} groups, and the C–O asymmetric stretching vibration, respectively (Brugnerotto et al., 2001; Chen et al., 2002; Shigemasa, Matsuura, Sashiwa, & Saimoto, 1996). In the spectrum of gelatin, the absorption bands at 3320, 3085 were assigned to the N–H stretching of amide(II), while those at 2960, 1640, 1540 and 1240 cm^{-1} for gelatin were assigned to C–H stretching of alkyl group, C=O stretching of amide (I) and N–H bending of amide(II), N–H bending of amide(III), respectively (Kim, Nimni, Yang, & Han, 2005).

In the hybrid hydrogels, the –OH stretching vibration band of CM-chitosan at 3300 cm^{-1} shifted to a higher wavenumber and the peak intensity increased as the gelatin was incorporated into CM-chitosan, confirming the formation of intermolecular hydrogen bonds between the amide groups and carboxyl groups in the hybrid gels. The N–H bending peak at 1540 cm^{-1} and C=O stretching peak at 1640 cm^{-1} became narrow and incisive, which indicated that the hydrogen bond formed inside gelatin was interrupted with the incorporation of CM-chitosan. The N–H stretching vibration peak of CM-chitosan shifted from 1540 to 1560 cm^{-1} when the gelatin/CM-chitosan ratio reached 40/60 and the peak density increased, whereas the density of 1060 cm^{-1} C–O stretching peak decreased, which further proved the formation of intermolecular hydrogen bonds between the carboxyl and amide groups of CM-chitosan and the gelatin (Kim et al., 2005). Therefore, the gelatin and CM-chitosan have excellent miscibility to create a uniform structural semi-IPN hydrogel network after hybrid and radiation crosslinking.

3.5. *Morphology*

The porous microstructure of the scaffolds had significant influence on the ability of the cells intrusion, proliferation and function in tissue engineering (Kasahara et al., 2003). Fig. 5 shows the SEM images of the lyophilized hydrogels, confirming that the porous hydrogel scaffolds have open and interconnected pores structure. The pore sizes and the thickness of the wall varied with the ratio of gelatin/CM-chitosan. With the addition of the CM-chitosan into the gelatin, the swelling capacity of the hydrogels markedly increased, resulting in the increase of the pore diameters and the decrease of the wall thickness. For the hybrid samples, the pore diameter distribution was between 15 and 40 μm, which is suitable for the permeation of nutrients, oxygen, and waste from the cells. What's more, the pores structure will support several other functions for wound healing materials. For instance, the promotion of drainage, prevention of exudates build-up, and the preparation of an optimal wound bed for autografting (Hong, Jin, Park, Ahn, & Kim, 2008).

3.6. *In vitro enzyme degradation*

Controllable biodegradation rate plays crucial roles in regulating cell proliferation and tissue regeneration (Kasahara et al., 2003). In addition to in vitro degradation in PBS, the enzyme-induced degradation behavior of gelatin/CM-chitosan hydrogels in lysozyme and collagenase was investigated. Lysozyme is the main enzyme in wound exudation fluid that could hydrolyze the $\beta(1 \rightarrow 4)$ glycosidic linkage in chitin and CM-chitosan (Hjerde, Varum, Grasdalen, Tokura, & Smidsrod, 1997). The hydrogel samples were incubated at 37 °C in lysozyme solution with a concentration of 0.4 mg/ml, which was close to the concentration of lysozyme in typical wound exudation fluid with inflammation (Frohm et al., 1996). Fig. 6(a) shows the dynamic degradation degree of the hydrogels after incubation in lysozyme solution at different times. With the increase of the CM-chitosan content, the degradation rate increased. Samples 5# and 6# were thoroughly digested by lysozyme within 2 days, while the other samples were completely degraded over a period of 3–5 days.

Being a protein contained scaffold, the collagenase as well as other protein enzymes will take part in the in vivo degradation of the hydrogels. Therefore, the in vitro collagenase induced degradation of gelatin/CM-chitosan hydrogels was investigated to allow a more comprehensive understanding of their biodegradability. Due to the extremely low concentration of collagenase in the real exudation fluid, a typical concentration of 0.1 mg/ml was chosen for the biodegradability study of collagen or gelatin based biomaterial (Park, Park, Kim, Song, & Suh, 2002; Pek, Spector, Yannas, & Gibson, 2004).

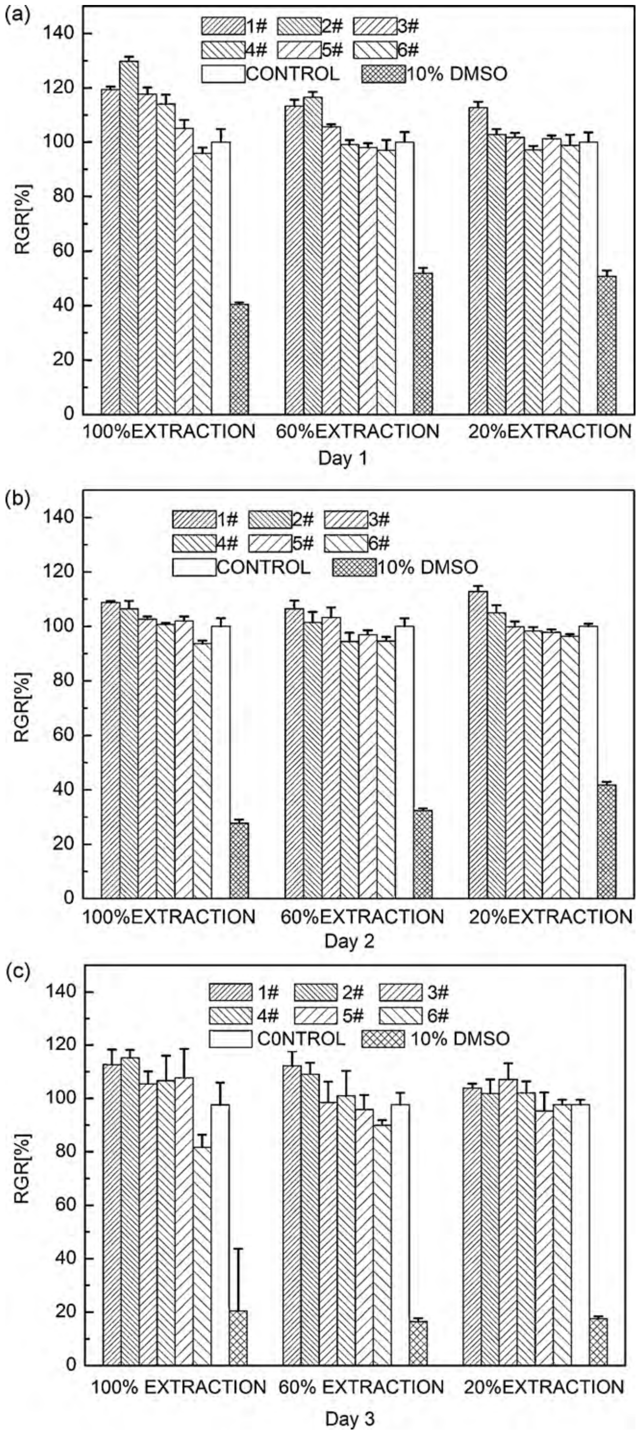

Fig. 7. Relative growth rate (RGR) of L929 in gelatin/CM-chitosan hydrogels extraction medium after incubated for 1 day (a), 2 days (b) and 3 days (c), respectively. The hydrogels were prepared at 30 kGy and the weight ratios of gelatin to CM-chitosan were 10:0(1#), 8:2(2#), 6:4(3#), 4:6(4#), 2:8(5#) and 0:10(6#), respectively.

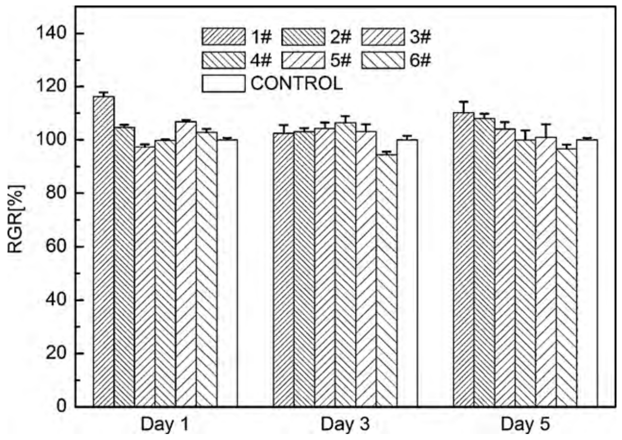

Fig. 8. Cell proliferation profile of NIH 3T3 cell on gelatin/CM-chitosan hydrogels after incubated for 1, 3 and 5 days. The hydrogels were prepared at 30 kGy and the weight ratios of gelatin to CM-chitosan were 10:0(1#), 8:2(2#), 6:4(3#), 4:6(4#), 2:8(5#) and 0:10(6#), respectively.

Fig. 6(b) shows the weight loss of the gelatin/CM-chitosan hydrogel in collagenase solution. Hydrogels with a higher ratio of gelatin/CM-chitosan, i.e. samples 1#–4, underwent faster degradation than those of sample 5#–6#. The samples 1#–4 were completely degraded with in 10 h in this protein-specific enzyme, while less than 30% of the 5# and 6# hydrogels were degraded after immersing in the collagenase solution for 40 h. According to the study on human skin, the presence of collagenase has a significant effect on the degradation of hydrogels with higher content of gelatin, therefore, promote the loss of gel (Stricklin, Bauer, Jeffrey, & Eisen, 1977; Woolley, Glanville, Roberts, & Evanson, 1978). The degradation ratio of 6#, which was slightly higher than 5#, was ascribed to its higher swelling capacity, which led to the disintegration of gel structure upon enzyme solution diffusion. The initial enzymatic digestion rate slightly increased in the sequence of sample 2# > 1# > 3# > 4#, and the abnormal sequence of the 1# and 2# samples can be attributed to the faster diffusion of the solution into the hydrogel network and thinner porous walls of 2# than the 1# sample.

3.7. *In vitro cell study*

3.7.1. *Cytotoxicity of hydrogel extract*

Cytotoxicity is one of the most important properties for biomaterials. The cytotoxicity of the gelatin/CM-chitosan hydrogels was evaluated following the guideline of ISO 10993-5 by incubating L929 cells with the extracts of the hydrogels. The RGRs of L929 after an incubation period of 1, 2 and 3 days were shown in Fig. 7, indicating that the RGRs of all the samples were higher than 95%, which could be classified to a scale 0 (non-toxicity). The excellent cytocompatibility of the hydrogels was ascribed to the biocompatibility of the gelatin and the CM-chitosan, as well as a green fabrication approach of the hydrogels, proving that the radiation crosslinking is a promising method to synthesize biomaterials. Among the samples with different composition, the RGRs of the gelatin-rich hydrogels were slightly higher than those of CM-chitosan-rich hydrogels. This result can be interpreted by the inhibition effect to fibroblast proliferation when the concentration of CM-chitosan was higher than a critical point (ca. 1000 ppm).

3.7.2. *NIH 3T3 cell proliferation on hydrogels*

In order to assess the potential of cell proliferation and following tissue regeneration on the hydrogels, the growth profile of NIH 3T3 fibroblasts on the hydrogel films was investigated by MTT assay (Fig. 8). On most of the hydrogel samples, NIH 3T3 fibroblasts grew faster than on the TCP and proliferated continuously from the first day to the fifth day. The result indicates that the fibroblasts could attach and proliferate well on the hydrogel surface. On the fifth day, the cell density on the hydrogels tended to decrease with the increase in the CM-chitosan fraction, which was caused by the extraction of CM-chitosan from the hydrogel to culture media. In terms of their high RGRs, the gelatin/CM-chitosan hydrogels displayed excellent cytocompatibility in both extracts and contact forms. Besides their biocompatibility, as discussed in this section, gelatin/CM-chitosan hydrogels were completely degradable in lysozyme, collagenase, and PBS. Also, they possess excellent swelling ability, uniform micro-porous structures and biomimic mechanical properties, which support their prospect for the application as skin scaffold and wound healing materials.

4. Conclusion

Gelatin/CM-chitosan hybrid hydrogels were prepared using a green fabrication method, i.e. radiation-induced-crosslinking. The hydrogels had excellent and adjustable water retention capacity (10–700 g/g dry gel) and a similar compressive modulus with that of soft tissue (10–200 kPa). Due to the promising miscibility between gelatin and CM-chitosan molecules, scaffolds with homogeneous semi-IPN networks and inter-connect porous structures can be achieved after crosslinking and lyophilizing. These hybrid hydrogels have improved flexibility, antimicrobial and water absorption capacity than gelatin hydrogels; meanwhile, they have superior handleability and mechanical properties than CM-chitosan hydrogels. The in vitro degradation study revealed that the hydrogels can be completely hydrolyzed by PBS, lysozyme, and collagenase solutions, which indicates their excellent and controllable biodegradability. Cell culture experiments demonstrated that the gelatin/CM-chitosan hybrid hydrogels had good cytocompatibility with a cytotoxicity of grade 0, and NIH 3T3 fibroblast cells could grow actively on the hydrogels. The comprehensive results of this study suggest their potential in the application of wound healing materials.

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