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An improved complex gel of modified gellan gum and carboxymethyl chitosan for chondrocytes encapsulation

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ABSTRACT

Gellan gum is a candidate material for cartilage tissue engineering, but its application is limited by the high gelation (Coutinho et al., 2010) temperature and lack of mechanical strength. In this study, oxidized gellan gum was prepared and mixed with carboxymethyl chitosan to form a double-network complex hydrogel with significantly improved gelation temperature ($T_{gelation}$) and mechanical properties. The $T_{gelation}$ was lowered from 42 °C to below physiological temperature by oxidation, and further reduced by complexing with carboxymethyl chitosan. The complex hydrogel showed an increased compressive modulus of 278 kPa, and an ability to return to the original shape after release of the compressive load. In vitro chondrocytes encapsulation and proliferation experiments found that the complex hydrogel significantly enhanced the viability of the cells. These results suggest that, the complex gel of gellan gum and carboxymethyl chitosan is a promising material for cartilage tissue engineering.

1. Introduction

Damaged articular cartilage frequently leads to a marked decrease in the quality of life due to its limited capacity for self repair (Risbud & Sittinger, 2002). Tissue engineering has been proposed as a new method to address problems such as organ failure and tissue degeneration (Chen, Harding, Ali, Lyon, & Boccaccini, 2008; Chong & Chang, 2006; Kim et al., 2010). In cartilage tissue engineering (Balakrishnan & Banerjee, 2010), cells are seeded on biomaterial scaffolds and then implanted into the defect in the host, where new functional tissue is formed, remodeled and integrated with the host. Hydrogels have gained significant attention as candidate materials for cartilage tissue engineering scaffolds because they can be processed into performs with similar three-dimensional shapes and initial mechanical strength similar to natural extracellular matrix due to their ability to retain great quantities of water (Drury & Mooney, 2003; Hoffman, 2002).

In the past decade, great progress has been achieved in the development of hydrogels for tissue engineering scaffold (Drury & Mooney, 2003). Various natural and synthetic hydrogel biomaterials have been processed using different techniques, such as alginate (Kuo & Ma, 2001), chondroitin sulfate (Chan, Caron, & Orth, 2007), hyaluronic acid (Wang et al., 2007), collagen (Liu, Xia, & Czernuszka, 2007). Each material has its own favorable properties as well as limitations, and the major limitations of hydrogels include their insufficient mechanical performance and relative harsh gelation conditions for cell encapsulation (Oliveira & Reis, 2010).

Gellan gum is a linear, anionic extracellular polysaccharide with repeating tetrasaccharide units of D-glucose, D-glucuronic acid, D-glucose, and L-rhamnose (Jansson, Lindberg, & Sandford, 1983; Moorhouse, Colegrove, Sandford, Baird, & Kang, 1981). Gellan gum can be conveniently processed into hydrogels that are resistant to heat and acid. It has been well exploited for applications in food and pharmaceutical industries (Deasy & Quigley, 1991; Jansson et al., 1983; Sosa-Herrera, Berli, & Martínez-Padilla, 2008). Recently, gellan gum has been investigated as a candidate material for tissue engineering because of its biocompatibility and low cytotoxicity (Silva-Correia et al., 2011). Oliveira et al. (2010) first studied gellan gum as a new biomaterial for cartilage regeneration. However, its application in tissue engineering is limited by its relatively high gelation temperature and insufficient mechanical strength. Generally, the form of a gellan gum hydrogel depends on temperature and cation (Tang, Tung, & Zeng, 1996), it exists as random coils at a high temperature, and the addition of cations (Na^+, K^+, Ca^{2+}) can introduce ionic bonds between the carboxyl groups of gellan and thus results in thermo-reversible gelation when cooled to below $T_{gelation}$. Usually, the $T_{gelation}$ of unmodified gellan is too high (>42 °C) for cell encapsulation (Coutinho et al., 2010). To reduce the $T_{gelation}$, Gong et al. (2009) adjusted the molecular weight of gellan by a chemical scissoring process via a $NaIO_4$-based oxidative cleavage reaction (Gong et al., 2009). In addition, gellan-based gels are typically too hard and brittle for successful application as tissue engineering

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scaffolds. Therefore, Nakayama et al. (2004) prepared a hydrogel with a double-network structure consisting of gellan gum and bacterial cellulose, and attained an improved elasticity compared with the gellan gel (Nakayama et al., 2004). However, even with the modified structure, the hydrogel still involves a long gelation time (1 week) and requires the use of crosslinking agents (e.g. EDC) that are toxic to cells. We hypothesized that introducing oxidation to gellan and a double-network into gellan gum hydrogel may overcome these limitations.

Chitosan is a natural cationic polymer comprised of (1 → 4)-glucosamine and N-acetyl-D-glucosamine units. It is biodegradable and biocompatible, and usually has a high molecular weight and strong network of hydrogen bonds (Di Martino, Sittinger, & Risbud, 2005; Jollès & Muzzarelli, 1999). However, chitosan itself has a very slow degradation rate and poor solubility in water (Muzzarelli, 1988). In addition, chitosan cannot be straight forwardly mixed with gellan gum because they would form insoluble polyelectrolyte complexation. Carboxymethyl chitosan (CM-chitosan) is a derivative of chitosan that has been widely studied for biomedical applications, such as controlled drug release and tissue engineering (Kim et al., 2008; Lu et al., 2007; Muzzarelli, 1982). CM-chitosan also has a good water solubility that could easily interact with other materials without pH variation (Muzzarelli, Ilari, & Petrarulo, 1994). Importantly, with amino groups on its chains that can readily react with aldehyde groups (Fu, Huang, Zhai, Li, & Liu, 2007), CM-chitosan forms double-network structure with oxidized gellan (Weng, Romanov, Rooney, & Chen, 2008).

In this study, we oxidized the adjacent dihydroxyl groups in gellan gum with $NaIO_4$ to lower its gelation temperature. In addition, as too many aldehyde groups may be toxic to cells, we further combined oxidized gellan with CM-chitosan to obtain a complex hydrogel through the Schiff-base reaction. We will show that the technique can improve the cellular compatibility and also enhance the mechanical property of the gel.

2. Experimental

2.1. Materials

Gellan gum (G1910) was purchased from Sigma Chemical Co. Carboxymethyl chitosan (M_w = 100,000) was purchased from Zhejiang Aoxin Biotechnology Co. The degree of acetylation was above 90%. The carboxymethyl substituted chitosans both on the hydroxyl oxygen atoms and nitrogen atoms of the glucosamine units, and the total degree of carboxymethylation was 81.5%. Other materials were obtained from Chengdu Kelong Chemical unless stated otherwise.

2.2. Synthesis of oxidized gellan gum

Oxidized gellan gum (O-GG) was synthesized by reacting plain gellan gum (P-GG) with sodium periodate. Briefly, 1 g of P-GG was dissolved in 100 mL of deionized water at 90 °C for 20–30 min to obtain a homogeneous aqueous solution. After solution cooled to room temperature, $NaIO_4$ (0.05 M) was then added at different dosages (3, 6, 7.5 and 9 mL) with gentle stirring and the solution was allowed to react for 4 h to attain various degrees of oxidization. An equimolar amount of ethylene glycol was added at predefined reaction time points to stop the oxidation reaction. After reaction for 30 min, the resulting solution was purified by dialysis for at least 3 days against distilled water to remove the excess $NaIO_4$, then frozen at −20 °C overnight and lyophilized at −80 °C, vacuum value 15 Pa for 2 days (VivTis Advantage plus EL-85). After that, the freeze-dried product was stored in a vacuum dryer.

2.3. Characterization of oxidized gellan gum

The obtained O-GG was analyzed by ^{1}H NMR (AVANCE II 600 MHz, Bruker CO. Switzerland) and intrinsic viscosities tests. We also determined the actual oxidization degrees compared with the theoretical oxidization degrees. First, 0.1 g of O-GG was dissolved in 20 mL of distilled water, and the solution pH was adjusted at room temperature to 5 by adding 0.1 M HCl. Second, 20 mL of hydroxylamine hydrochloride solution (0.1 M) was adjusted to pH 5 by adding 0.5 M NaOH. These two solutions were then mixed and allowed to react for 15 min. The resulting liquid was then titrated with 0.5 M NaOH to pH 5 and the volume of NaOH consumed was recorded. A blank titration was done and served as control (Bryant, Arthur, & Anseth, 2005; Li et al., 2006). The oxidization degrees were calculated by the following equation:

$$(\text{Degree of oxidation, DO})\% = \frac{645(V_1 - V_2) \times c \times 10^{-3}}{2M}$$

V_1 is the volume of NaOH reacted by oxidized gellan, mL; V_2 the volume of NaOH reacted by blank response, mL; c the concentration of NaOH, mol/L; M the exact mass of sample, g; and 645 is the monomer molecular weight of gellan gum residues.

2.4. Preparation of hydrogels

The lyophilized O-GG and P-GG were both dissolved in deionized water at 2.5% (w/v) under constant stirring at 90 °C for 30 min. Afterwards $CaCl_2$ was added into the solution at a final $CaCl_2$ concentration of 0.1% (w/v) to produce hydrogels.

The complex gellan gum hydrogel (C-GG) was prepared by adding CM-chitosan to the O-GG solution with $CaCl_2$ at 90 °C. The final concentration of CM-chitosan solution was 1% (w/v). The hydrogel was obtained by cooling the solution to room temperature.

2.5. Characterization of hydrogels

2.5.1. Gelation point test

The gelation point test of hydrogels was measured following previous studies (Gong et al., 2009). Briefly, 0.5 mL of various gellan solutions (approximately 90 °C) were added into centrifuge tubes and then incubated in water bath with accurate temperature monitoring. The temperature of water bath was decreased by 1 °C every 20 min. The initial temperature of the water bath was kept higher than $T_{gelation}$ to prevent gelation of the samples. The tubes (n = 5) were brought for observation after the temperature has become stable every 10 min. When the gellan solutions lose their flow ability, the current temperature was recorded as the gelation temperature.

2.5.2. Swelling and degradation kinetics

To study the swelling kinetics of the modified hydrogels, the samples (about 0.1 g) were immersed in 1.5 mL of phosphate buffered saline (PBS) at 37 °C. At different time points, the hydrogels (n = 3) were removed from the solutions and blotted with a filter paper to absorb excess water on surface. Their wet weights were measured (w_t) and compared with the initial wet weight (w_0). The swelling ratio (SR) was calculated according to the following equation:

$$\text{SR} = \frac{w_t}{w_0}$$

The degradation experiments were performed by incubating the hydrogels in PBS at 37 °C and monitoring their weight loss. At each time point, the samples (n = 3) were removed from the

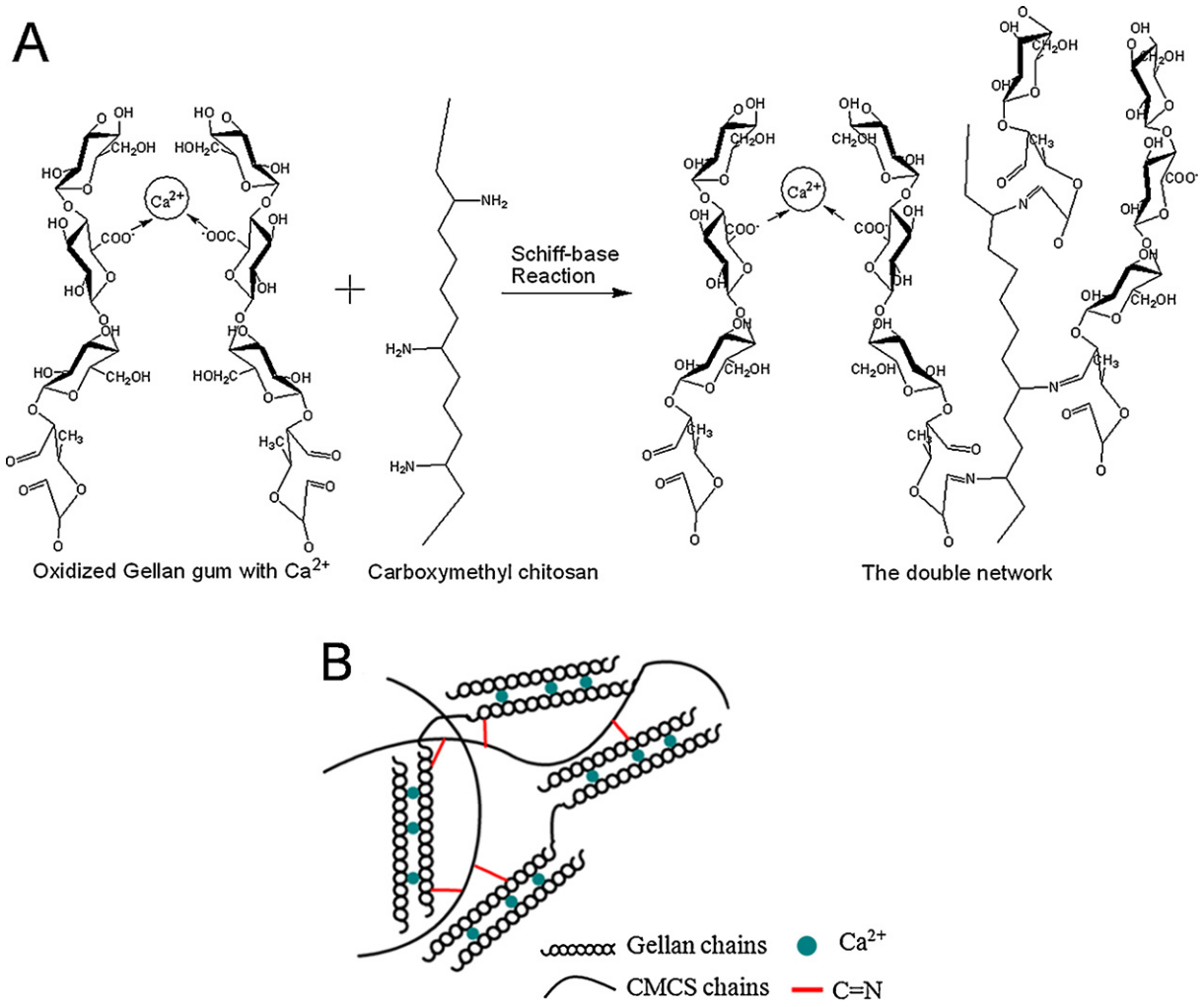

Fig. 1. Schiff-base formation between amino groups of CM-chitosan and aldehyde groups of oxidized gellan gum (A). In gellan chains, cis-dihydroxyl of rhamnose was oxidized to dialdehyde, the addition of Ca^{2+} introduced ionic bonds between the carboxyl groups of gellan via electrostatic interaction, subsequently aldehyde groups and amino groups of CM-chitosan formed the second network via the Schiff-base reaction. The crosslinking mechanism of complex hydrogel (B). Gellan gum chains formed double helix conformations with Ca^{2+}, and then CM-chitosan chains link the aldehyde zones to the formation of a three dimensional network, that created the gel.

solution, freeze dried and then weighed to determine the weight of the remaining mass.

2.5.3. Mechanical properties

To assess the effect of the degree of oxidation and hydrogel type on the mechanical properties of the hydrogels, the compressive stress/strain measurement were performed with a mechanical tester (CMT4104, Shenzhen SANS Testing Machine Co. Ltd.). Generally, freshly prepared hydrogels were molded in 48-well culture plates into pellets (17 mm thick, 10.8 mm in diameter, $n = 3$) and compressed at a rate of 1 mm/min to 50% of their original height. The compressive modulus was defined as the slope of the linear region of the stress–strain curve in the 5–15% of the strain range.

2.5.4. Scanning electron microscopy

The morphology of freeze-dried P-GG, O-GG(3) and C-GG(3) hydrogel scaffolds were studied by field emission scanning electron microscopy (FE-SEM, S-4800, HITACHI, Tokyo, Japan). Pore sizes of the lyophilized scaffolds were measured from SEM micrographs using Image Pro Plus 6.0 software (Media Cybernetics, USA).

2.5.5. Cell culture

Chondrocytes isolated from articular cartilage of newborn rabbits were used to evaluate the material biocompatibility. Generally, newborn rabbits were sacrificed by pentobarbital over-dose. Pieces of cartilage were minced and washed with PBS and then digested with 0.1% (w/v) collagenase type II solution (Gibco) in PBS for 6 h at 37 °C, then centrifuged at 1500 rpm for 5 min to obtain a cell pellet. Following removal of supernatant, cells were resuspended in plastic culture dishes and incubated in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C using Dulbecco's minimum essential medium (DMEM) with 10% FBS. All experimental procedures involving animals were approved with the Animal Care and Use Committee of Sichuan University.

2.5.6. Cell proliferation test

To determine the cell proliferation of the modified gellan, chondrocytes were incubated on the hydrogels. O-GG(3) and C-GG(3) gels were air-dried to form membranes, and placed in 24-well plate, then chondrocytes were seeded on each sample for 1 mL at a density of 1×10^4 cells/mL. As a control, parallel experiments were carried out in P-GG simultaneously. The proliferation of chondrocytes on the samples was determined by MTT

assay [3-(4,5-dimethylthialzol-2-yl)-2,5-diphenyltetrazolium bromide] after culture for 1, 3 and 7 days.

2.5.7. Cell encapsulation

For cell encapsulation, the procedure was the following. P-GG, O-GG(3) and C-GG(3) solution was prepared as described previously and kept their temperature slightly higher than $T_{gelation}$ for cell encapsulation. Then, the hydrogel solutions mixed with chondrocytes was injected into 24-well culture plates (350 μL per well) with a cell density of 2×10^6 cells/mL. After the gelation of hydrogels, every well was added 1 mL medium. The culture medium was changed every other day. The morphology of the cells at 1, 3 and 7 days was observed by confocal laser scanning microscopy (CLSM, Leica SP5, Leica Microsystems, Wetzlar, Germany) using FDA (fluorescein diacetate) and PI (propidium iodide) for "live/dead" dye staining.

2.6. Statistical analysis

All results were expressed as mean ± standard deviation (SD). Data were analyzed by using one- and two-way analyses of variance (ANOVAs). Tukey's multiple comparison test and Bonferroni post-hoc test were used with one-way and two-way ANOVA each to determine significance between specific treatments. For all statistical tests, the level of significance was set at $^*p < 0.05$.

3. Results and discussion

In this study, complex hydrogels were synthesized by modified oxidation gellan and CM-chitosan and their physicochemical property was characterized for further chondrocytes culture. Firstly, the oxidation reaction cleaved polymer chains into smaller segments, leading to the decreasing in polymer molecular weight and the increasing of cross-linking aldehyde groups. This allowed for the formation of two entangled networks of different crosslinked polymers as described in Fig. 1A. In the first step, gellan gum chains were connected with Ca^{2+}, and then the random coils formed double helix conformations when temperature was lowered to $T_{gelation}$ (Takahashi et al., 2004). The presence of cation was essential to the formation of a stable hydrogel via electrostatic interaction (physical crosslinking). In the second step, a complex gel was formed by a chemical crosslinking reaction (Schiff-base formation) between the pendant amino groups of CM-chitosan and the aldehyde group in the oxidized gellan. This was followed by the assembly of helices into oriented bundles called junction zones. Regions of ionically crosslinked gellan gum chains at the ends of the helices then linked C=N zones together to form a double network as shown in Fig. 1B.

The oxidation reaction cleaved polymer chains into smaller segments, leading to the decrease in polymer intrinsic viscosity and the increasing of cross-linking aldehyde groups. Intrinsic viscosity values of plain and oxidized gellan are in Table 1. Compared with plain gellan gum, the [η] of oxidized gellan was reduced significantly and progressively with the dosage of $NaIO_4$, indicating the decrease of molecular weight of gellan gum by the cleavage of the polymer chains. The actual oxidization degree was inversely related to [η] and closed to the theoretical oxidization degrees. The

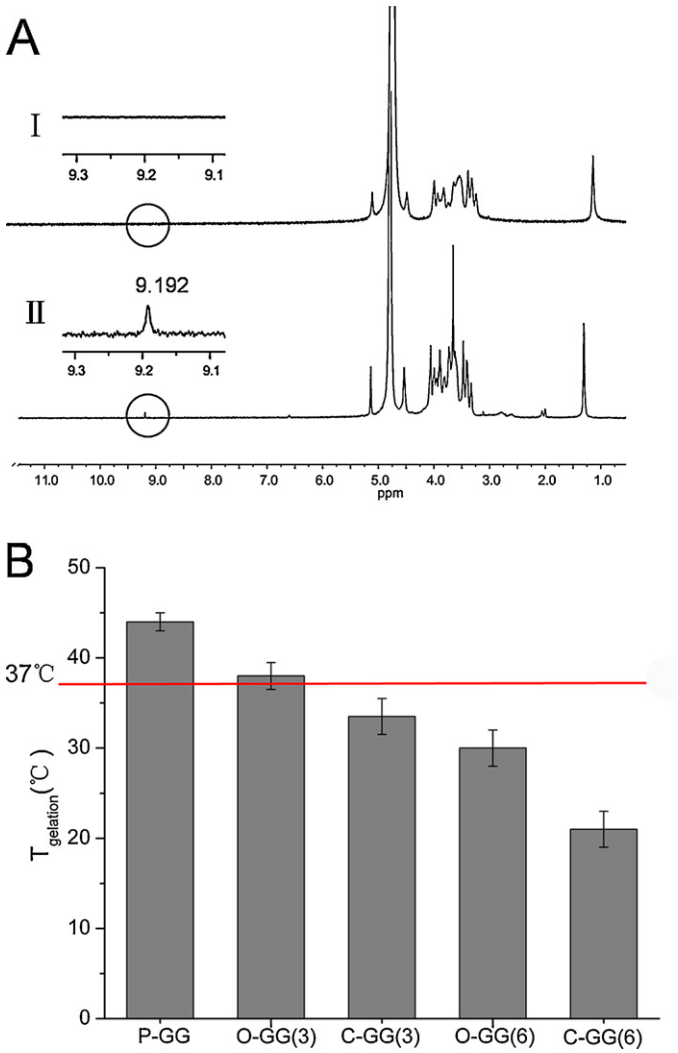

Fig. 2. (A) ^{1}H NMR spectra (600 MHz, in D_2O) of (I) P-GG and magnified spectrum at 9.2 ppm, (II) O-GG(3) and magnified spectrum at 9.2 ppm. (B) Gelation temperature of P-GG, O-GG(3), C-GG(3), O-GG(6) and C-GG(6) ($n = 5$).

NMR spectrum of O-GG(3) (Fig. 2A) shows a characteristic peak at 9.192 ppm, confirming furtherly the creation of aldehyde groups in the oxidized gellan gum.

Fig. 2B shows the gelation temperatures of various gellan solutions. P-GG had a very high gelation temperature of 44 °C at a concentration of 2.5% (w/v). After oxidation, the gelation temperature of gellan was decreased by the increasing of $NaIO_4$. For example, the gelation temperature of O-GG(3) was 38 °C, while the gelation temperature of O-GG(6) was 25 °C. The oxidization process partial destroyed the crosslinking points, hence gelation became difficult and the gelation temperature decreased. Thus, O-GG(9) failed to gelate because of excessively high oxidization degree. It was notable that the $T_{gelation}$ further decreased with the addition of CM-chitosan in oxidized gellan, especially for C-GG(6). This can be

Table 1
The intrinsic viscosity and actual oxidization degree of oxidized gellan gum with different oxidant dosages.

Sample	Theoretical oxidization degree (%)	Dosages of $NaIO_4$ (mL)	Actual oxidization degree (%)	Intrinsic viscosity [η] ($L\,g^{-1}$)
P-GG	0	0	0	19.6
O-GG(3)	10	3	9.7	17.7
O-GG(6)	20	6	18.6	15.5
O-GG(7.5)	25	7.5	23.2	14.9
O-GG(9)	30	9	36.2	11.3

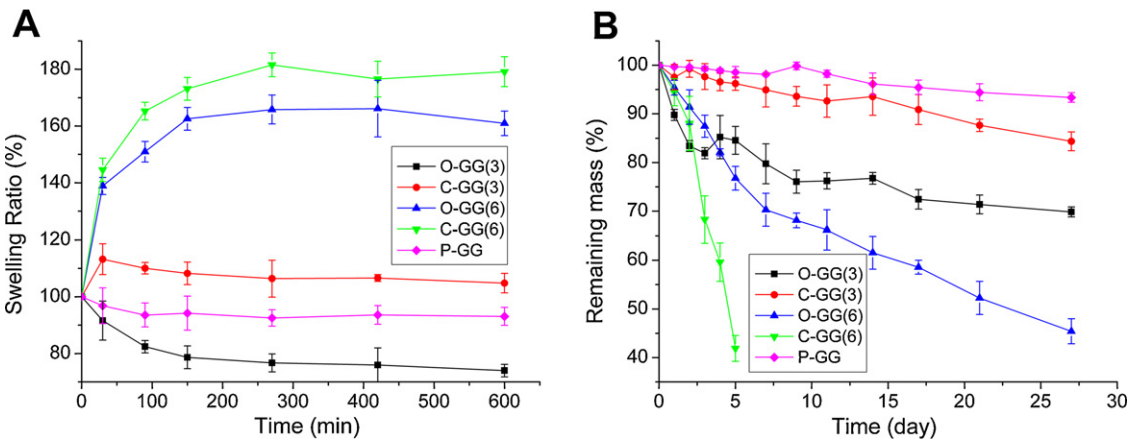

Fig. 3. Swelling (A) and degradation (B) kinetics of hydrogels in PBS at 37 °C ($n = 3$).

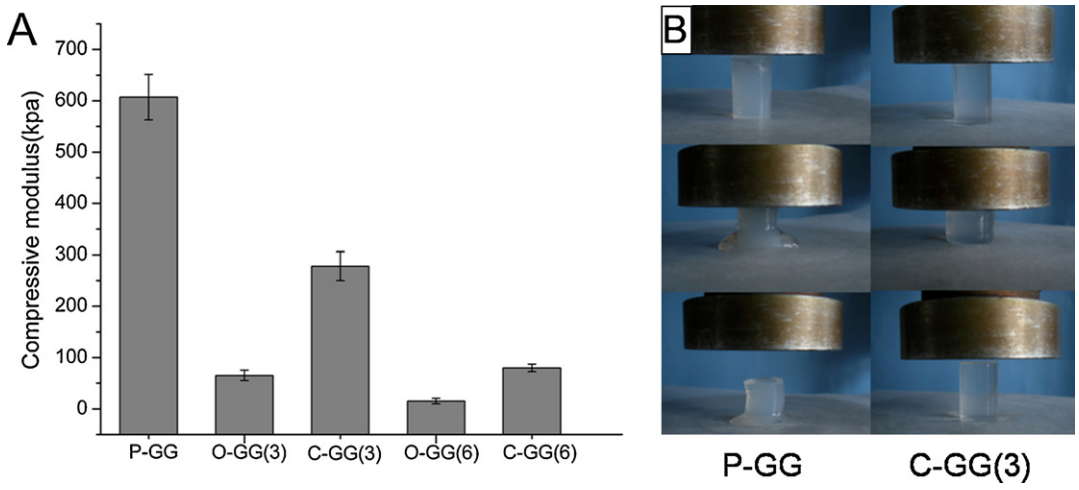

Fig. 4. The compression modulus for P-GG, O-GG(3), C-GG(3), O-GG(6) and C-GG(6) hydrogels (A), there were significant differences among all groups($n = 3$; $^*p < 0.05$); photographs of P-GG (left) and C-GG(3) (right) hydrogels during the compression test (B) (upper: before compressing; middle: in compressing; lower: after compressing).

explained that the electrostatic interaction between gellan chains was inhibited by CM-chitosan, resulted in $T_{gelation}$ decreased (Lin, Liang, Chung, Chen, & Sung, 2005).

Fig. 3 depicted the swelling and degradation behavior of hydrogels in PBS. O-GG(6) and C-GG(6) presented the highest swelling ratio of almost 180%, while P-GG, O-GG(3) and C-GG(3) showed little change and remained stable after 100 min. Moreover, the swelling behaviors of O-GG(3) and O-GG(6) were completely different; O-GG(3) reduced 20% of weight while O-GG(6) increased 80%. Generally, the swelling ratio was influenced by the crosslinking density, gel composition, network structure, etc. (Durmaz & Okay, 2000; Liu & Chan-Park, 2009). The results showed CM-chitosan had little effect on water absorbency while oxidation showed obvious effect. For gellan gum, higher extent of oxidization leaded to lower crosslinking density. Therefore, the swelling ratios of C-GG(3), O-GG(6) and C-GG(6) hydrogel were much higher than P-GG. CM-chitosan also had positive effect on the swelling behavior under the same oxidation conditions as the curves of C-GG(3) and C-GG(6) shown, contributed by total concentration increased (Abreu, Bianchini, Forte, & Kist, 2008).

The degradation of five hydrogels was conducted by monitoring their weight lost in PBS (Fig. 3B). The crosslinking density and mechanism had great influence on its degradation properties. The curves of P-GG, O-GG(3) and O-GG(6) indicated that hydorgels

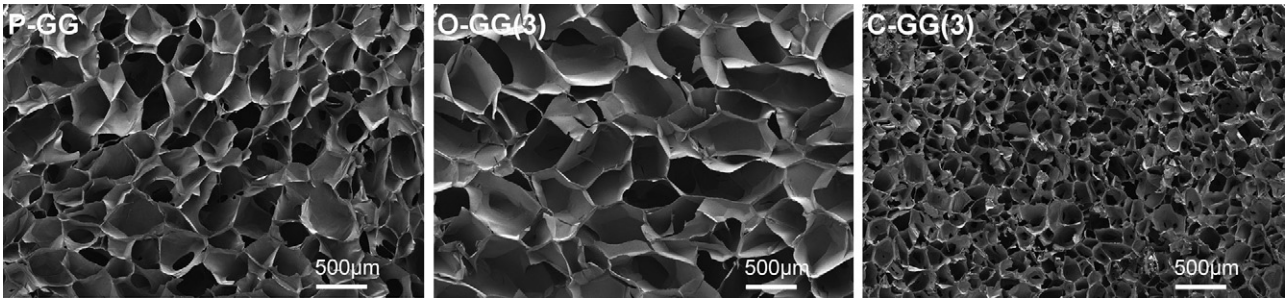

Fig. 5. SEM images of cross section of hybrid scaffold P-GG, O-GG(3) and C-GG(3).

with lower degrees of oxidation were more stable against degradation. As was discussed before, crosslinking density decreased with the increase of oxidation extent thus resulting in a higher degradation behavior. Also by this reason, both P-GG and C-GG(3) experienced slow degradation, with 93% and 84% mass remaining after 27 days, respectively, while C-GG(6) rapidly disintegrated after immersion in PBS for 5 days because of excessive oxidation as well as O-GG(6). In PBS, C-GG(3) degraded slower than O-GG(3) due to the addition of CM-chitosan. Specifically, the physical crosslinking combined with chemical crosslinking mechanisms prolonged the rate of degradation when compared to only physically crosslinked hydrogels, additionally, it is reported that the degradation rate of chitosan is relatively slow (Kim et al., 2008), thus in the same oxidation extent, C-GG was more stable than O-GG.

P-GG hydrogel showed a generally excellent compressive strength of 607 kPa (Fig. 4A), but the samples did not resume their original shape after compression tests (Fig. 4B left). O-GG was softer and brittle; C-GG(3) was elastic and could quickly return to its original shape after tests (Fig. 4B right). O-GG(3), C-GG(3), O-GG(6) and C-GG(6) exhibited compressive modulus of 65, 278, 15 and 79 kPa (Fig. 4A), respectively. There were significant differences among all groups. It was obvious that the compressive modulus decreased with the increasing dosage of $NaIO_4$. Also, under the same oxidation conditions complex hydrogels had significantly better mechanical properties than oxidized hydrogels. This varied

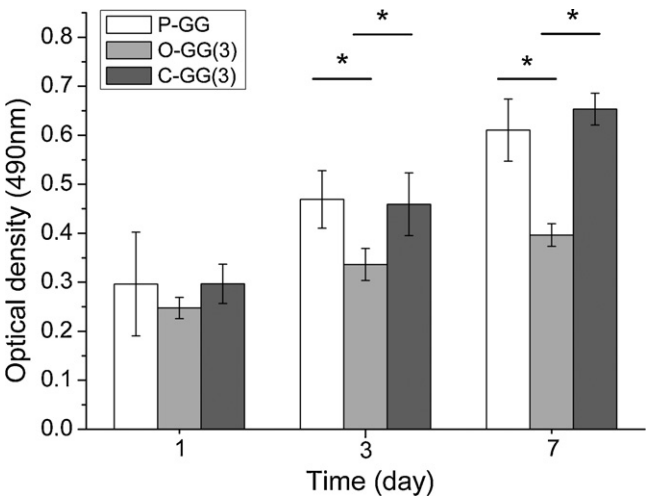

Fig. 6. Cell proliferation assessed by MTT test performed 1, 3 and 7 days after seeding ($n = 3$; $^*p < 0.05$).

mechanical strength was corresponding with the hydrogel network structure. As shown by SEM of the cross-sectional morphologies of the lyophilized hydrogels in Fig. 5. All samples contained interconnected and homogeneously distributed pores, but with obviously

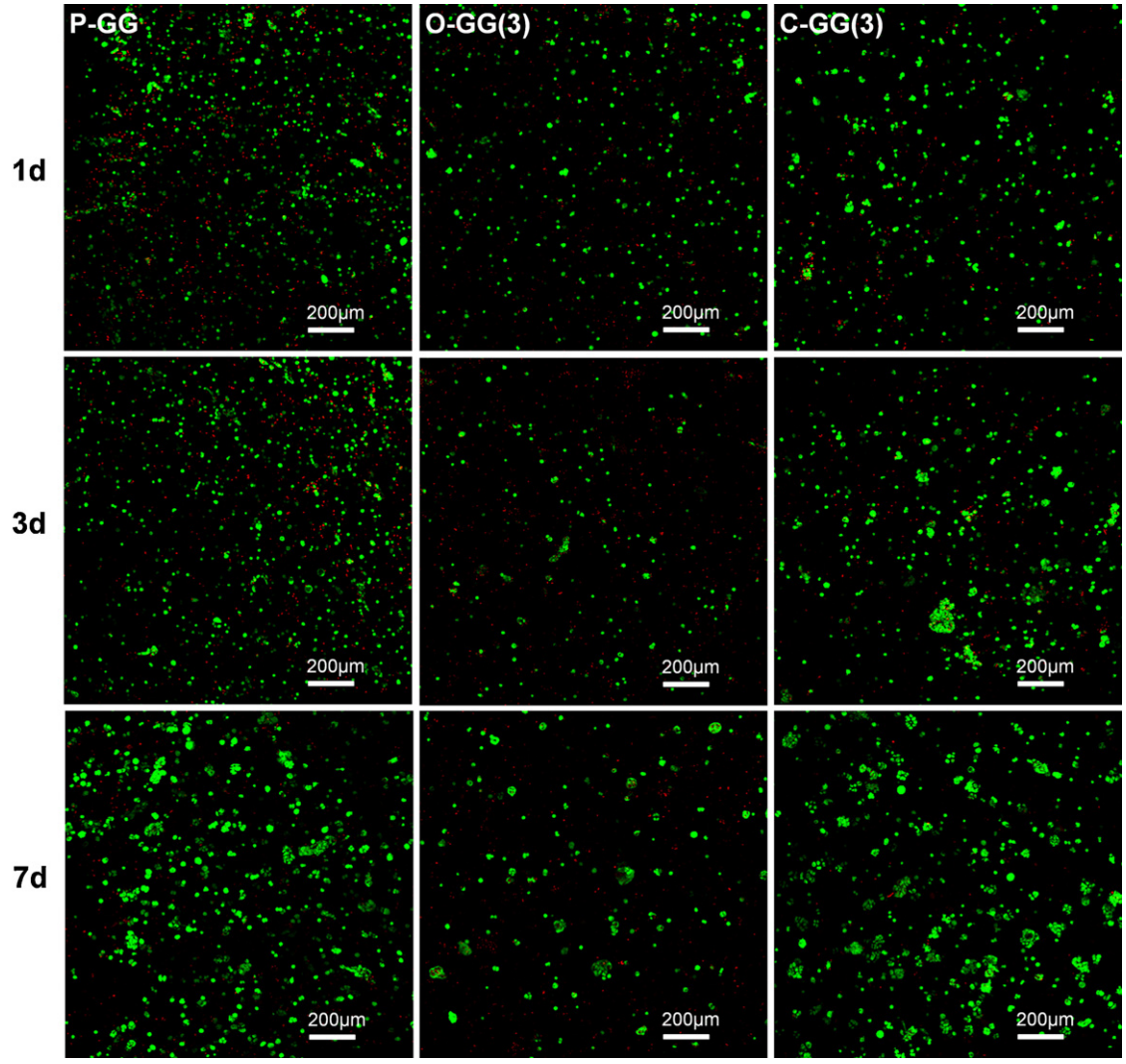

Fig. 7. Fluorescence micrographs of live (FDA, green) and dead (PI, red) chondrocytes in hydrogels cultured in vitro for 1, 3 and 7 days.

different pore sizes. P-GG exhibited a pore size of 369 ± 57 μm, and O-GG(3) had a slightly larger pore size of at 479 ± 66 μm. C-GG(3) had the smallest pore size of 198 ± 32 μm and was more compact than P-GG and O-GG(3). There was no obvious phase separation in C-GG(3). Obviously, O-GG(3) hydrogel had a porous structure with larger pores, the loose network might result in a high brittleness. Conversely, the structure of C-GG(3) was significantly more compact with homogeneously distributed small and uniform pores. This was attributable to the crosslinking density and material composition (Xiao, Zheng, Xiao, Fan, & Zhang, 2011). This compact and uniformly structure contributed to a higher strength. Complex hydrogel was formulated by developing double network structures with gellan and CM-chitosan as shown in Fig. 1B, and this double network structure would increase the strength of hydrogels (Zhang, Qadeer, & Chen, 2011).

The cell viability of chondrocytes in hydrogels was firstly evaluated by MTT as shown in Fig. 6. The number of viable cells in all samples increased with culturing time. There were no significant differences in optical density among P-GG, O-GG(3) and C-GG(3) at 1 day (0.296 ± 0.106, 0.247 ± 0.022 and 0.0297 ± 0.040, respectively). After being cultured for 3 and 7 days, significant differences were found between P-GG and O-GG(3) as well as O-GG(3) and C-GG(3), and the optical density of O-GG was obvious lower than the other two groups. P-GG and C-GG(3) presented little difference in optical density during the whole culture process.

Fig. 7 depicted chondrocytes residing in the P-GG, O-GG(3) and C-GG(3) hydrogels after being cultured for 1, 3 and 7 days. Most of the living chondrocytes exhibited a round shape and could be homogeneously encapsulated within the hydrogels after culture. Slightly, more dead cells were found in P-GG and O-GG(3) at 1 and 3 days, and even in O-GG(3) after 7 days' culture. The cell number was consistent with the results of MTT, and the O-GG hydrogel contained the fewest cells. Overall, both P-GG and C-GG(3) hydrogels significantly enhanced cell viability. Aldehyde groups in O-GG(3) are potentially cytotoxic, however, cell still proliferated slowly in O-GG(3). In the same time, cells proliferated in C-GG(3) as well as in P-GG hydrogels from 1 to 7 days. The results suggest that aldehyde groups did have a potential disadvantage influence on cell viability; however, after complexing with CM-chitosan, the aldehyde groups were partially eliminated thus the potential diverse effect was reduced. Moreover, chitosan itself has a structure similar to that of glycosaminoglycans and shows promise for cartilage repair as previously reported to Bhardwaj et al. (2011), so the cell showed higher proliferation in C-GG(3) than that in O-GG(3).

4. Conclusion

In this work, complex gellan gum hydrogel was synthesized by modified oxidation gellan and CM-chitosan. The double network structure was created by Ca^{2+} crosslinking and Schiff reaction. The results were quite promising in terms of hydrogel gelation temperature and mechanical strength. The $T_{gelation}$ was reduced from 42 °C to below physiological temperature after oxidation and further decreased with carboxymethyl chitosan. The C-GG(3) hydrogel showed high compression modulus of 278 kPa, which will return to the initial state as soon as the load is canceled. Swelling and degradation behavior of the hydrogels were dependent on the oxidation extent and CM-chitosan concentration. By adjusting these parameters, it was possible to provide the hydrogels with a very large variety of swelling and degradation behavior. P-GG and C-GG(3) showed lower cytotoxicity than O-GG(3) and consequently chondrocytes proliferation on P-GG and C-GG(3) was more rapidly than on O-GG(3). In summary, the complex hydrogel scaffold may be useful for cartilage tissue engineering.

Acknowledgements

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