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Analysis of freeze-gelation and cross-linking processes for preparing porous chitosan scaffolds

Chien-Yang Hsieh, Sung-Pei Tsai, Ming-Hwa Ho [1], Da-Ming Wang, Chung-En Liu, Cheng-Hsuan Hsieh, Hsien-Chung Tseng, Hsyue-Jen Hsieh *

Department of Chemical Engineering, National Taiwan University, Taipei 10617, Taiwan, ROC

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Abstract

A newly developed freeze-gelation method for fabricating porous chitosan scaffolds for tissue engineering applications was analyzed. The influences of three process variables of freeze-gelation method on the tensile properties of the scaffolds were investigated. It was found that a higher freezing temperature (−20 °C) and concentration of acetic acid (1–2 M) significantly increased the tensile stress and strain of the scaffolds at maximum load, whereas a higher ethanol concentration (95%) in the rinse buffer slightly increased the tensile stress of the scaffolds at maximum load. Further, we successfully established a correlation to quantify the influence of these process variables of the freeze-gelation method on the tensile properties of the scaffolds. Hence, the correlation can be used for quantitative predictions of the tensile properties of the scaffolds. To enhance the tensile strength, the scaffolds were cross-linked using glutaraldehyde (GTA), *N*-(3-dimethyl-aminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), or tripolyphosphate (TPP). We found that both the tensile stress and strain at maximum load significantly and dose-dependently increased with the addition of the cross-linking agent, GTA. However, the addition of the cross-linking agents, EDC and TPP, only moderately increased the tensile stress and strain at maximum load, but no dose response was observed. In summary, our study analyzed the freeze-gelation and cross-linking processes for fabricating porous chitosan tissue engineering scaffolds with different tensile properties.
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Keywords: Chitosan; Freeze-gelation; Cross-linking; Tensile property

1. Introduction

Tissue engineering provides a novel way to recover physiological function by seeding cells onto scaffolds constructed of natural or artificial materials, together with the use of growth factors and other signaling molecules to modulate cell proliferation and differentiation. Hence, the fabrication of tissue engineering scaffolds is an important topic in biomaterial research. Porous scaffolds are generally needed because of their high specific surface areas (Hou, Tsai, Liu, & Feng, 2003; Keller, Collins, Niederauer, & McGee, 1997; Lange et al., 2004; Langer & Vacanti, 1993; Murphy, Kohn, & Mooney, 2000; Murphy & Mooney, 1999; Peter, Miller, Yasko, Yaszemski, & Mikos, 1998; Thomson et al., 1999).

Chitosan is a polysaccharide constituted of *N*-glucosamine and *N*-acetyl-glucosamine units, in which the number of *N*-glucosamine units exceeds 50%. Chitosan is positively charged and solubilized by protonation of its amino groups when the solution pH is <6 (Chatelet, Damour, & Domard, 2001; Khor & Lim, 2003; Koyano, Minoura, Nagura, & Kobayashi, 1998; Madihally & Matthew, 1999; Muzzarelli, 1977; Muzzarelli, Jeuniaux, & Gooday, 1986). Chitosan can be degraded into nontoxic products *in vivo*, and thus it has been widely used in various biomedical applications (Chatelet et al., 2001; Kratz et al., 1997; Lee, Ha, & Park,

* Corresponding author. Tel.: +886 2 23633097; fax: +886 2 23623040.
E-mail address: hjhsieh@ntu.edu.tw (H.-J. Hsieh).
[1] Present address: Department of Chemical Engineering, National Taiwan University of Science and Technology, Taipei 10607, Taiwan, ROC.

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1995; Miyazaki, Ishii, & Nadai, 1981; Rhoades & Roller, 2000; Roller & Covill, 1999). In this study, we fabricated porous chitosan scaffolds using a newly developed freeze-gelation method (Ho et al., 2004). In this method, a chitosan solution is frozen and then immersed in a gelation environment at a temperature lower than the freezing point of the chitosan solution. Our previous studies (Ho et al., 2004; Hsieh, Tsai, Wang, Chang, & Hsieh, 2005) showed that the freeze-gelation method is both more energy-efficient and time-efficient, producing less residual solvent, and is easy to scale up. According to our experience, when utilizing the freeze-drying method to fabricate chitosan scaffolds, acetic acid remains in the scaffolds even after a 3-day incubation in the freeze-drying process. Thus, the scaffolds will redissolve when immersed in pure water. On the other hand, the freeze-gelation method we used took only 24 h, and no residual acetic acid was detected. Therefore, the freeze-gelation method was chosen for fabricating porous chitosan scaffolds. However, this newly developed method has not been intensively investigated by adjusting such process variables as the freezing temperature, the concentration of acetic acid, and the concentration of ethanol in the rinse buffer.

For biomedical applications, the tensile properties of scaffolds are very important. There are several process variables in the freeze-gelation method that can influence the tensile properties of scaffolds. For example, during the freezing process, because the temperature is sufficiently low to allow freezing of the solution, phase-separation occurs and leads to the formation of ice and chitosan-rich phases (Han, 2000; Ho et al., 2004). In principle, the porous structure of the scaffolds is determined at the phase-separation stage (Ho et al., 2004). So the freezing temperature of the chitosan solution is an important process variable. At the gelation stage, the frozen, chitosan-rich phase is precipitated, and the space occupied by the ice later becomes pores. However, in our study, the chitosan solution was prepared using acetic acid as the solvent, and NaOH solution was used for the gelation process. Neutralization of the acid and base is an exothermic process, which can result in the melting of the frozen scaffolds locally, although the environmental temperature was below the freezing point of the chitosan solution. Local melting might lead to local destruction of the porous structure. Therefore, the concentration of acetic acid in the chitosan solution, which affects the extent of local melting, is another important process variable. After the gelation stage, the porous scaffolds were immersed in a rinse buffer containing ethanol in order to fix and harden the porous scaffolds. The ethanol concentration in the rinse buffer is also an important process variable.

In this study, the influence of these three important process variables (freezing temperature, concentration of acetic acid, and ethanol concentration) on the tensile properties of the scaffolds (tensile stress and strain) were determined. The results indicated that a higher freezing temperature and a higher concentration of acetic acid increased the tensile stress and strain of the scaffolds. A higher ethanol concentration in the rinse buffer also slightly increased the tensile stress. We also established correlations to quantify the influence of these three process variables on the tensile properties of the scaffolds. The correlations provide quantitative predictions of the tensile properties of the scaffolds. To further enhance the mechanical strength of the scaffolds, we also cross-linked the scaffolds individually using glutaraldehyde (GTA), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), and tripolyphosphate (TPP). GTA is a chemical cross-linking agent that mediates a reaction that occurs between two amino groups (Dawson et al., 1983). EDC is also a chemical cross-linking agent, and the cross-linking reaction occurs between an amino group and a carboxyl group (Olde Damink et al., 1996). TPP is an ionic cross-linking agent with a negative charge (Aral & Akbuga, 1998). The enhancement in the tensile properties of the chitosan scaffolds after adding these cross-linking agents was determined.

2. Materials and methods

2.1. Materials

Chitosan (with a M.W. of about 4×10^5 and a degree of deacetylation of 90%) was purchased from Taiwan Chitin Chitosan Co. (Taipei, Taiwan). Other chemicals of the highest purity available were used and were purchased from Sigma–Aldrich (St. Louis, MO, USA), Biochrom AG (Berlin, Germany), Biological Industries (Kibbute, Israel), or GIBCO Invitrogen (Grand Island, NY, USA). Tissue culture flasks and 12-well plates were obtained from Corning (Schiphol-Rijk, The Netherlands). The Micro BCA protein assay kit was purchased from Pierce (Rockford, IL, USA).

2.2. Preparation of porous scaffolds by freeze-gelation method

The advantages of the freeze-gelation method are described above in the Section 1. To prepare the porous chitosan scaffolds, chitosan powder was first added to water with stirring to ensure that the powder was uniformly distributed in the water. Then a certain amount of acetic acid (HAc) was added to the solution with stirring for 12 h until the chitosan had dissolved. The prepared solution was centrifuged for 15 min at $3000 \times g$ for degassing. The solution was then poured into dishes and frozen for 12 h at various freezing temperatures. The frozen samples were immersed in a 3 M NaOH/ethanol solution at −20 °C for 12 h, followed by rinsing with rinse buffer (ethanol solution) for 12 h. The scaffolds were then washed using a phosphate-buffered saline (PBS) solution.

2.3. Preparation of cross-linked porous scaffolds

Three cross-linking agents including glutaraldehyde (GTA, 0.005–0.02%), *N*-(3-dimethylaminopropyl)-*N*′-ethyl-

carbodiimide hydrochloride (EDC, 0.5–5%), and tripolyphosphate (TPP, 0.1–0.5%) were used to prepare cross-linked chitosan scaffolds. Cross-linking agents were directly added to the scaffold solutions with stirring for 12 h. Then the freeze-gelation method was employed to prepare the porous scaffolds. The freezing temperature was −80 °C and the rinse buffer was a 95% ethanol solution.

2.4. Influence of the freezing temperature

To investigate the influence of freezing temperature on the tensile properties of the scaffolds, scaffold solutions with 4 wt% of chitosan and 0.2 M HAc were prepared and used to fabricate scaffolds by the freeze-gelation method. Four temperatures (−20, −40, −60, and −80 °C) were chosen for freezing the scaffold solution. The rinse buffer was a 95% ethanol solution.

2.5. Influence of the concentration of acetic acid in the scaffold solution

To understand the influence of the concentration of acetic acid, scaffold solutions with 4 wt% chitosan were prepared for fabricating scaffolds by the freeze-gelation method. We chose five different concentrations (0.2, 0.5, 0.8, 1.0, and 2.0 M) of an acetic acid solution to prepare the scaffold solution. The freezing temperature was −80 °C and the rinse buffer was a 95% ethanol solution.

To investigate the influence of the acid equivalent, three sets of experiments were carried out. Chitosan was dissolved in 0.2 M HAc + 0.3 M HCl (acid equivalent of 0.5 M), 0.2 M HAc + 0.8 M HCl (acid equivalent of 1.0 M), or 0.2 M HAc + 1.8 M HCl (acid equivalent of 2.0 M) solutions. The freezing temperature was −80 °C and the rinse buffer was a 95% ethanol solution.

2.6. Influence of the ethanol concentration in the rinse buffer

To investigate the influence of the ethanol concentration in the rinse buffer, scaffold solutions containing 4 wt% chitosan and 0.2 M HAc were prepared to fabricate scaffolds by the freeze-gelation method. Three different compositions containing ethanol concentrations of 0%, 50%, and 95% of rinse buffers were chosen to rinse the scaffolds. The freezing temperature was −80 °C.

2.7. Establishment of predictive correlations

To analyze the process for preparing the scaffolds, we established correlations to describe the system. The tensile properties (stress and strain) of the scaffolds were prepared under the conditions (freezing temperature of −80 °C, concentration of acetic acid of 0.2 M, and ethanol concentration in the rinse buffer of 95%) as the reference values, and these were used to normalize the other tensile property values. Quadratic or linear polynomial functions were used to fit the data. We thus obtained three equations which described the relations between the normalized tensile property values and the freezing temperature, the concentration of acetic acid, and the ethanol concentration in the rinse buffer, respectively. We then used these three equations to obtain estimated values of the tensile properties and compared them with the experimental values under different operating conditions (see Section 2.8).

2.8. Validation of the predictive correlation

To evaluate the correlations established in Section 2.7, a series of experiments was carried out. Three process variables were investigated: the freezing temperature, the concentration of acetic acid in the scaffold solution, and the ethanol concentration in the rinse buffer, respectively, whose effects on the tensile properties of scaffolds were investigated in Sections 2.4–2.6. Values for each variable and combinations of the process variables are listed in Table 1.

2.9. Analysis of the tensile properties of the scaffolds

The tensile properties of the porous scaffolds were determined using a tensile strength instrument (model LRX, Lloyd, Hampshire, UK). The data of tensile properties were gathered and calculated using the NEXYGEN 4.0 (Lloyd) software. The test used was a general-purpose pull-to-break test. The prepared scaffolds were swollen in PBS for 2 h and cut into a specific dog-bone shape (6 cm long, 2 cm wide at the ends, and 1 cm wide in the middle). The thickness of each individual scaffold was measured. The thickness of the scaffolds was about 3.0 mm. But the thicknesses of the scaffolds made by the scaffold solutions with higher acid concentrations were obviously thinner (at about 1.7–2.0 mm) than the others. The tensile analysis was performed at a stretching rate of 10 mm/min with a pre-load of 0.5 N to determine the tensile stress and strain at maximum load for each scaffold.

2.10. Analysis of the scaffold structure by SEM

To investigate the microstructure, cross-sections of the porous scaffolds were observed using scanning electron microscopy (SEM, model JEOL JSM-6300, Hitachi, Tokyo, Japan). For the SEM analysis, scaffold samples were rinsed in a 95% ethanol solution at −20 °C for 12 h and then dried in a lyophilizer. Samples were coated with gold–palladium and then examined by SEM.

3. Results and discussion

3.1. Influence of the freezing temperature

To investigate the influence of changes in the freezing temperature on the tensile properties, scaffolds were examined using a tensile strength instrument (Fig. 1). The tensile stress of the scaffolds at maximum load increased as the

Table 1
The values and combinations of the three process variables (freezing temperature, concentration of acetic acid (HAc) in chitosan solution, and concentration of ethanol in the rinse buffer) for validating the predictive correlations

Process condition	Freezing temp. (°C)	Conc. of HAc in chitosan solution (M)	Conc. of ethanol in the rinse buffer (v%)
A	−80	0.2	95
B	−80	0.2	0
C	−80	0.8	95
D	−80	0.8	0
E	−20	0.2	95
F	−20	0.2	0
G	−20	0.8	95
H	−20	0.8	0

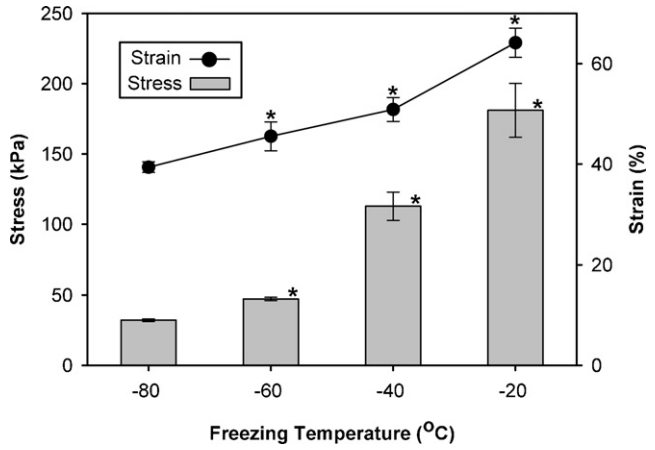

Fig. 1. Tensile properties (stress and strain at maximum load) of porous chitosan scaffolds frozen at different temperatures ($n \geqslant 6$, means ± SD). The fabrication conditions were 4 wt% chitosan in a 0.2 M acetic acid solution, frozen at a certain temperature, gelled in a 3 M NaOH/ethanol solution at −20 °C, and rinsed with a 95% ethanol solution. Significance (t-test) is indicated: $*p < 0.005$ vs. the −80 °C sample.

freezing temperature rose. When comparing scaffolds frozen at −80 °C with those at −20 °C, the stress increased by about 5-fold, from 32.13 (at −80 °C) to 181.11 kPa (at −20 °C) (Fig. 1). The tensile strain also presented a similar trend. The strain increased by about 0.6-fold, from 39.37% (at −80 °C) to 64.17% (at −20 °C).

The major effect of freezing temperature was on the pore sizes of the scaffolds. As mentioned in the Section 1, when phase-separation occurs during freezing, ice and chitosan-rich phases are formed. In the freeze-gelation process, the ice phase becomes pores, and the frozen chitosan-rich phase becomes the substance of the scaffold. The lower the freezing temperature is, the quicker the freezing rate of the chitosan scaffold solution is. At a faster freezing rate, the grains in the chitosan-rich phase have a shorter time to grow, and the sizes of the chitosan grains are smaller. The ice crystals are also smaller, so the pore sizes are smaller. Sizes of the chitosan grains directly influence the tensile properties of the scaffolds. Therefore, a freezing temperature of −20 °C generated porous chitosan scaffolds with larger-sized grains and pores that resulted in enhanced tensile properties (stress and strain).

For comparison, we also used the freeze-drying method to fabricate porous scaffolds. The process conditions were freezing at −80 °C, and drying at −50 °C for 4 days. The tensile stress and strain values of the freeze-dried chitosan scaffolds were 34.54 ± 1.04 kPa (stress) and 41.26 ± 2.03% (strain), which are similar to the tensile properties of scaffolds fabricated by the freeze-gelation method.

3.2. Influence of the concentration of acetic acid in the chitosan solution

To investigate the influence of the concentration of acetic acid in the chitosan scaffold solution on the tensile properties, the tensile stress and strain of the scaffolds at maximum load were examined using a tensile strength instrument (Fig. 2). The tensile stress of the scaffold increased dose-dependently with the concentration of acetic acid. When comparing the scaffolds prepared in 0.2 M acetic acid with those prepared in 2.0 M acetic acid, the tensile stress increased by about 3-fold, from 32.13 (at 0.2 M) to 137.59 kPa (at 2.0 M) (Fig. 2). The change in tensile strain was not obvious. The effect of the concentration of acetic acid is mainly due to the heat released from acid–base neutralization during the gelation stage. The frozen chitosan solution slightly remelts when put into the NaOH/EtOH solution. This local remelting causes the chitosan solution to react with the NaOH/EtOH solution in the liquid phase. Thus the deposits of chitosan become denser and more compact than those which do not undergo remelting. Data on the thicknesses of the scaffolds also support this hypothesis: with an increase in the concentration of acetic acid, the scaffolds became thinner. The quantity of heat released increased with the amount of acetic acid, so

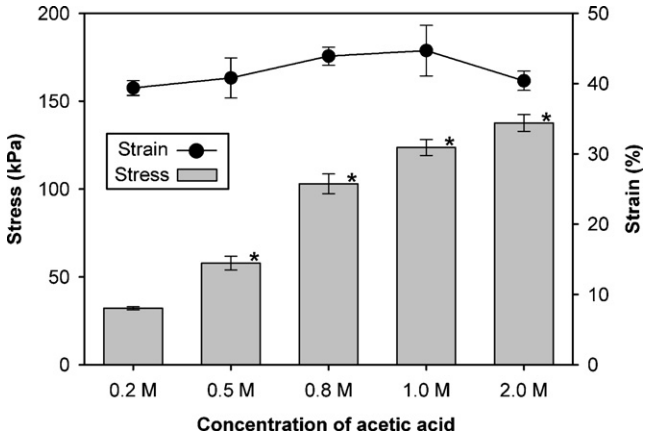

Fig. 2. Tensile properties (stress and strain at maximum load) of porous chitosan scaffolds prepared in acetic acid (HAc) solutions of different concentrations ($n \geqslant 6$, means ± SD). The fabrication conditions were 4 wt% chitosan in acetic acid solution, frozen at −80 °C, gelled in a 3 M NaOH/ethanol solution at −20 °C, and rinsed with a 95% ethanol solution. Significance (t-test) is indicated: $*p < 0.005$ vs. the 0.2 M HAc sample.

the tensile stress dose-dependently increased with the concentration of acetic acid. We propose that not just acetic acid but also other acids can trigger this remelting phenomenon.

Since the pH value of the scaffold solution may influence the freezing process and the size of the ice crystals in this system, we measured the pH value of each acetic acid solution. The pH values (concentrations) of the acetic acid solution were 3.02 (0.2 M), 2.61 (0.5 M), 2.52 (0.8 M), 2.44 (1.0 M), and 2.22 (2.0 M), respectively. Differences in the pH values in this system were not significant, so we propose that differences in pH values of various acetic acid solutions can be ignored.

To verify our proposal, we added 0.3–1.8 M hydrochloric acid (HCl) to the 0.2 M acetic acid (HAc) solution during scaffold preparation. The results are shown in Fig. 3. The data set of 0.2 M HAc + 1.8 M HCl is not shown because the scaffolds collapsed after the gelation process. The tensile stress still dose-dependently increased with the total acid equivalent, although the amount of acetic acid was the same. This result supports our proposal. However, with the same total acid equivalent, the more HCl there was, the higher the stress was, but the trend of strain was not obvious (by comparing Figs. 2 and 3). To explain this phenomenon, the exothermic rate has to be considered. The degree of dissociation of hydrochloric acid is higher than that of acetic acid, so the exothermic rate during neutralization is higher. This made the phenomenon of scaffold remelting more significant, so the tensile stress increased. However, the excessively high exothermic rate caused considerable remelting of the scaffolds, thus causing the collapse of the scaffolds prepared with 0.2 M HAc + 1.8 M HCl. We thought that the excessive remelting phenomenon made the scaffolds at least partially dense and compact. Therefore, they were not entirely porous scaffolds. Data on the thicknesses of the scaffolds also support this inference. Usually the strain of denser scaffolds was lower than that of porous ones. So the tensile strain decreased as the amount of hydrochloric acid increased.

3.3. Influence of the ethanol concentration in the rinse buffer

To investigate the influence of the ethanol concentration in the rinse buffer on the tensile properties, scaffolds rinsed in ethanol solutions of different concentrations were examined using a tensile strength instrument (Fig. 4). The tensile stress of the scaffolds at maximum load slightly increased with the ethanol concentration in the rinse buffer, but the tensile strain remained relatively constant.

Chitosan is not soluble in ethanol. Therefore, rinsing with an ethanol solution can promote the deposition of chitosan and thus stabilize the scaffold. We proposed that after the gelation stage, there is still a small amount of chitosan which is not yet deposited. Immersion of the scaffold in the ethanol solution may cause further deposition of the chitosan, but the primary structure of scaffolds is not changed. So the tensile stress only slightly increased with the ethanol concentration in the rinse buffer due to further chitosan deposition. The fact that the thickness of scaffolds rinsed with different concentrations of ethanol solution remained relatively constant also supports this hypothesis. Comparison of the results presented in Figs. 1–4 indicates that the influence of the ethanol concentration in the rinse buffer was not as significant as the freezing temperature and the concentration of acetic acid.

3.4. Establishing and validating the predictive correlations

By analyzing the data in Sections 3.1–3.3, we proposed a hypothesis that the influences of the three

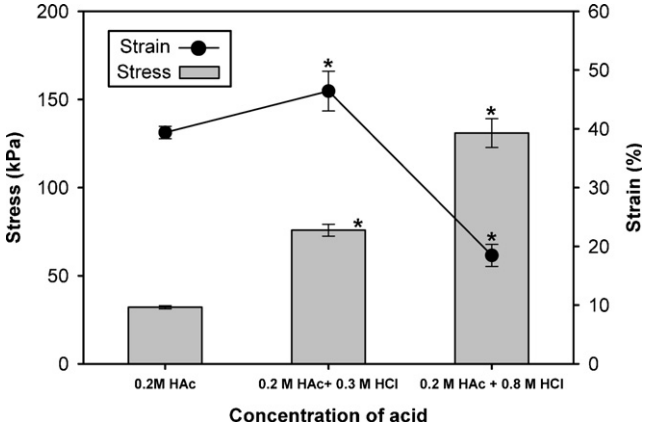

Fig. 3. Tensile properties (stress and strain at maximum load) of porous chitosan scaffolds prepared in solutions with the same concentration of acetic acid (HAc) but different concentrations of hydrochloric acid (HCl) ($n \geqslant 6$, means ± SD). The fabrication conditions were 4 wt% chitosan in hydrochloric acid and a 0.2 M acetic acid solution, freezing at −80 °C, gelation in a 3 M NaOH/ethanol solution at −20 °C, and rinsing with a 95% ethanol solution. Significance (*t*-test) is indicated: $*p < 0.005$ vs. the 0.2 M HAc sample.

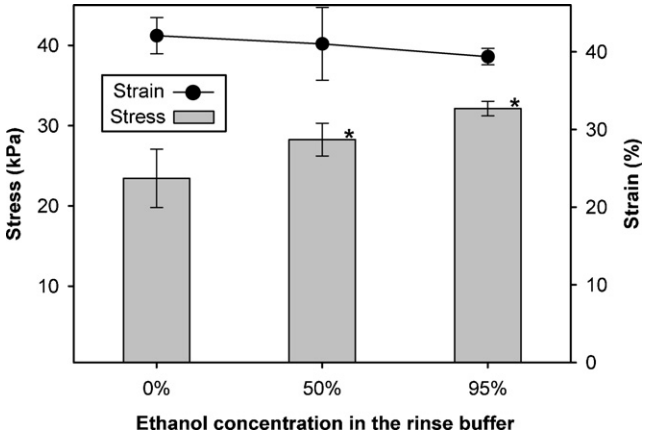

Fig. 4. Tensile properties (stress and strain at maximum load) of porous chitosan scaffolds prepared using rinse buffer containing different concentrations of ethanol (EtOH) ($n \geqslant 6$, means ± SD). The fabrication conditions were 4 wt% chitosan in a 0.2 M acetic acid solution, freezing at −80 °C, gelation in a 3 M NaOH/ethanol solution at −20 °C, and rinsing with rinse buffer. Significance (*t*-test) is indicated: $*p < 0.005$ vs. the 0% EtOH sample.

process variables are independent and can be multiplied together. According to this hypothesis, we established a correlation to quantitatively describe the influence of these three process variables on the tensile properties of the scaffolds at maximum load by the method described in Section 2.7. The results are shown in Table 2. Based on these results, we established equations of correlation to predict the tensile stress and strain at maximum load within our operating ranges (freezing temperatures of −20 to −80 °C, concentration of acetic acid of 0.2–1.0 M, and ethanol concentration in the rinse buffer of 0–95%):

$$\text{Stress (kPa)} = 32.13 \times (8.961 + 0.1831T_F + 0.001034T_F^2) \times (0.4250 + 2.489C_A - 1.002C_A^2) \times (0.7319 + 0.002851C_E)$$

and

$$\text{Strain (\%)} = 39.37 \times (1.776 + 0.0101T_F) \times (0.9590 + 0.1804C_A) \times (1.072 - 0.0007190C_E);$$

where T_F is the freezing temperature (°C), C_A is the value of concentration of acetic acid in scaffold solution (M), and C_E is the ethanol concentration in the rinse buffer (%).

To evaluate the established correlation, a series of experiments was carried out. The results are given in Table 3. The errors were almost smaller than 10%, indicating that the tensile properties could successfully be predicted within the operating ranges using this correlation. The success of the correlation revealed two points. First, it verifies the hypothesis: the influences of freezing temperature, concentration of acetic acid, and ethanol concentration in the rinse buffer are virtually independent and can be multiplied together. Sec-

Table 2
The establishment of correlations between the three process variables and the tensile properties (stress and strain at maximum load) of the scaffolds

(A) Tensile stress at maximum load

	Stress (kPa)	Normalized value (y)	Correlation equation
Freezing temperature			
T_F (°C)			
−80	32.13	1.000	$y = 8.961 + 0.1831 \times T_F + 0.001034 \times T_F^2$
−60	47.24	1.470	
−40	112.8	3.512	$r^2 = 0.9977$
−20	181.1	5.637	
Concentration of acetic acid			
C_A (M)			
0.2	32.13	1.000	$y = 0.4250 + 2.489 \times C_A - 1.002 \times C_A^2$
0.5	57.85	1.801	
0.8	103.0	3.206	$r^2 = 0.9986$
1	123.6	3.849	
Ethanol concentration in the rinse buffer			
C_E (v%)			
0	23.44	0.73	$y = 0.7319 + 0.002851 \times C_E$
50	28.26	0.88	$r^2 = 0.9989$
95	32.13	1.00	

(B) Tensile strain at maximum load

	Strain (%)	Normalized value (y')	Correlation equation
Freezing temperature			
T_F (°C)			
−80	39.37	1.000	$y' = 1.776 + 0.0101 \times T_F$
−60	45.53	1.156	
−40	50.86	1.292	$r^2 = 0.9984$
−20	64.17	1.630	
Concentration of acetic acid			
C_A (M)			
0.2	39.37	1.00	$y' = 0.9590 + 0.1804 \times C_A$
0.5	40.81	1.037	
0.8	43.91	1.115	$r^2 = 0.9723$
1	44.70	1.135	
Ethanol concentration in the rinse buffer			
C_E (v%)			
0	42.08	1.069	$y' = 1.072 - 0.0007190 \times C_E$
50	41.02	1.042	$r^2 = 0.9757$
95	39.37	1.000	

Table 3
The comparison of experimental values and estimated values of tensile stress and strain at maximum load of chitosan scaffolds prepared under different process conditions

Process condition	Stress (kPa)			Strain (%)		
	Est. value	Exp. value	Error (%)	Est. value	Exp. value	Error (%)
A	28.70	32.20	−11.94	38.06	39.38	−3.47
B	20.95	23.39	−11.87	40.65	42.08	−3.52
C	91.14	102.9	−13.02	42.20	43.90	−4.03
D	66.52	75.00	−12.75	45.07	48.74	−8.14
E	164.3	181.0	−10.14	61.89	64.16	−3.68
F	120.0	133.0	−10.88	66.10	68.94	−4.30
G	521.8	543.3	−4.11	68.62	73.14	−6.59
H	380.9	408.7	−7.29	73.29	77.44	−5.67

The estimated values were calculated from the correlation equations established in Section 3.4.

ond, the tensile properties of the scaffolds could be predicted within our operating ranges using this correlation, and therefore the correlation may be a great help in the design and fabrication of various chitosan scaffolds.

3.5. Analysis of the scaffold structure by SEM

The microstructure of a scaffold has a prominent influence on cell proliferation, function, and migration, all of which are key issues in tissue engineering. To investigate the influences of the three process variables on the microstructure, cross-sections of porous scaffolds fabricated under four different process conditions were demonstrated using SEM (Fig. 5).

The reason why we chose these four conditions was that they demonstrated the influence of the three process variables. When we compared conditions A with B, the difference was the ethanol concentration in the rinse buffer. As mentioned in Section 3.3, immersion of a scaffold in an ethanol solution might cause further deposition of chitosan, but the structure does not change. The SEM micrographs support this hypothesis: the microstructure in Fig. 5A is quite similar to that in Fig. 5B.

When we compared conditions A and C, the difference was the concentration of acetic acid. As mentioned in Section 3.2, the quantity of heat released increased with the concentration of acetic acid, so the deposits of chitosan would be denser and more compact than those which had not remelted. The SEM micrographs also support this hypothesis: the microstructure in Fig. 5C is more compact than that in Fig. 5A.

When we compared conditions A and E, the difference was the freezing temperature. As discussed in Section 3.1, the lower the freezing temperature, the quicker the freezing rate of the chitosan solution should be. At a quicker freezing rate, the grains in the chitosan-rich phase have a shorter time to grow, and the sizes of the grains should be smaller. The ice crystals also should be smaller, so the pore

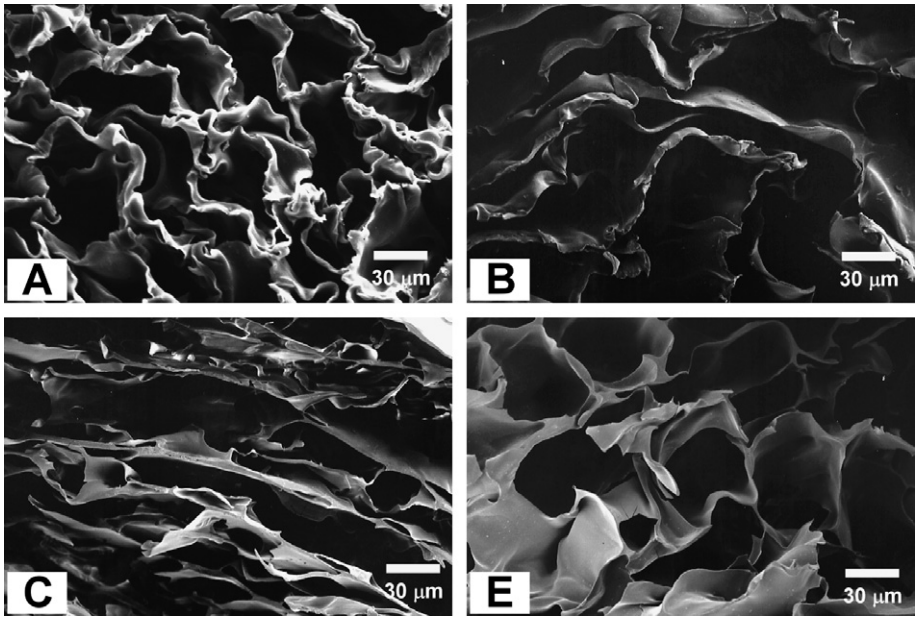

Fig. 5. SEM micrographs of cross-sections of chitosan scaffolds fabricated by the freeze-gelation method under different process conditions. (A, B, C, and E) represent the process conditions listed in Table 1 (Bar = 30 μm).

sizes are smaller. The SEM micrographs also support this hypothesis: the pore sizes in Fig. 5E are larger than those in Fig. 5A.

3.6. Effects of cross-linking agents

Cross-linked scaffolds were examined using a tensile strength instrument in order to investigate the influence of cross-linking agents (Figs. 6–8). For GTA cross-linked scaffolds, the tensile stress and strain at maximum load both dose-dependently increased with the addition of GTA (Fig. 6). For EDC cross-linked scaffolds, the tensile stress and strain at maximum load were both a little higher than the untreated control, but there was no obvious difference among different EDC concentrations (Fig. 7). For

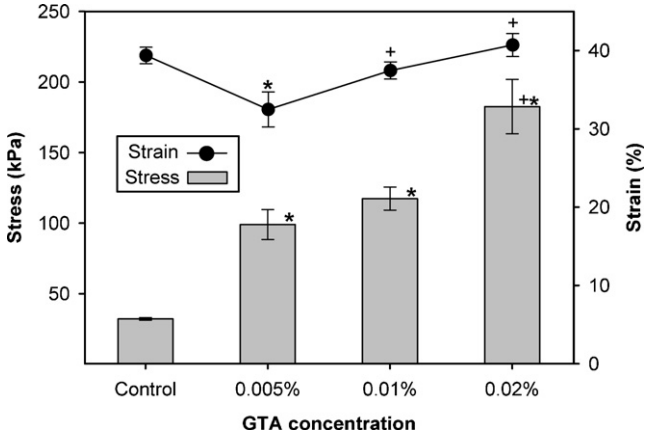

Fig. 6. Tensile properties (stress and strain at maximum load) of porous chitosan scaffolds cross-linked by GTA ($n \geqslant 6$, means ± SD). The fabrication conditions were 4 wt% chitosan in a 0.2 M acetic acid solution, freezing at −80 °C, gelation in a 3 M NaOH/ethanol solution at −20 °C, and rinsing with a 95% ethanol solution. Significance (t-test) is indicated: $^{*}p < 0.005$ vs. the control; $^{+}p < 0.05$ vs. the 0.005% GTA sample.

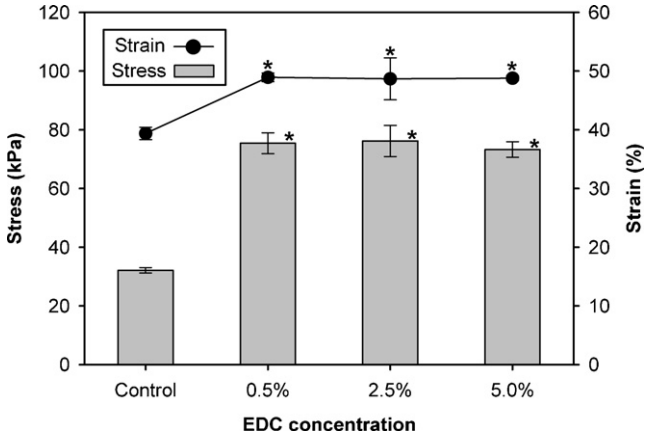

Fig. 7. Tensile properties (stress and strain at maximum load) of porous chitosan scaffolds cross-linked by EDC ($n \geqslant 6$, means ± SD). The fabrication conditions were 4 wt% chitosan in a 0.2 M acetic acid solution, freezing at −80 °C, gelation in a 3 M NaOH/ethanol solution at −20 °C, and rinsing with a 95% ethanol solution. Significance (t-test) is indicated: $^{*}p < 0.005$ vs. the control.

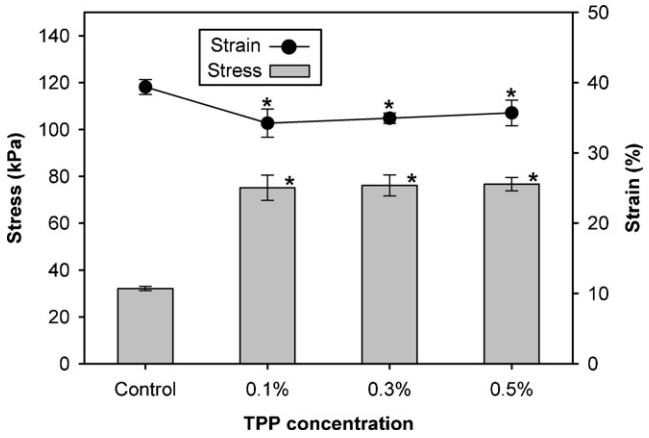

Fig. 8. Tensile properties (stress and strain at maximum load) of porous chitosan scaffolds cross-linked by TPP ($n \geqslant 6$, means ± SD). The fabrication conditions were 4 wt% chitosan in a 0.2 M acetic acid solution, freezing at −80 °C, gelation in a 3 M NaOH/ethanol solution at −20 °C, and rinsing with a 95% ethanol solution. Significance (t-test) is indicated: $^{*}p < 0.005$ vs. the control.

TPP cross-linked scaffolds, the tensile stress at maximum load was a little higher than that of the untreated control, but the tensile strain remained unchanged. There were also no obvious differences among the different TPP concentrations (Fig. 8).

There are two factors which affect the tensile properties of scaffolds. One is the nature of the material used, and the other is the structure of the scaffolds. In general, enhancement of the material nature should increase the tensile stress and reduce the tensile strain. GTA is a chemical cross-linking agent that mediates the reaction which occurs between two amino groups. Chitosan has amino groups, so it can be cross-linked by GTA. When comparing the scaffolds treated with 0.005% GTA and the control, it was obvious that the addition of GTA changed the material nature by forming covalent bonds between amino groups, so the tensile stress increased and the tensile strain decreased. However, when comparing the scaffolds treated with different concentrations of GTA, we found that both the tensile stress and tensile strain increased dose-dependently with GTA. To explain this phenomenon, the change in the thickness of the scaffolds should be addressed. With an increase in the concentration of GTA, the thickness of the scaffolds decreased, thus causing the structure of the scaffold to become more compact, and this may have been the cause of the increase in strain. EDC is also a chemical cross-linking agent, and the cross-linking reaction mainly occurs between an amino group and a carboxyl group. However, there is no carboxyl group on the chitosan chain. Therefore the cross-linking effect of EDC was not obvious for chitosan, and the tensile properties among different EDC concentrations were almost the same. TPP is an ionic cross-linking agent with a negative charge. Since chitosan is positively charged by protonation of the amino groups when the solution pH is below 6, the intermolecular electrostatic interaction contributes to the cross-linking effect.

Because the scaffolds were treated with a NaOH solution which neutralizes the positive charges on the amino groups of the chitosan, we propose that there might be only a small amount of positive charge still present on the chitosan chain. So the intermolecular electrostatic interaction between chitosan and TPP is thought to be weak, and thus the cross-linking effect was not obvious.

4. Conclusions

We analyzed the freeze-gelation method and cross-linking process to fabricate porous chitosan scaffolds. Analyses of the process variables indicated that a higher freezing temperature and concentration of acetic acid in the scaffold solution increased the tensile stress and strain of the scaffolds at maximum load, while a high ethanol concentration in the rinse buffer only slightly increased the tensile stress of the scaffolds. We also established a correlation to predict the tensile properties of the scaffolds in operating ranges of the three process variables. To enhance the tensile properties, the scaffolds were cross-linked by GTA, EDC, or TPP. We found that both the tensile stress and strain at maximum load dose-dependently increased with the addition of GTA. The tensile stress of scaffolds treated with EDC or TPP was a little higher than that of the control, but there was no obvious dose-dependence. In short, our study analyzed the freeze-gelation and cross-linking processes. We also established a correlation to predict the tensile properties of the scaffolds. This correlation should be helpful in the design and fabrication of various chitosan scaffolds for tissue engineering applications. Further research can be carried out on factors such as the freezing speed and the heat transfer behavior in the freeze-gelation process. Moreover, we plan to apply the freeze-gelation method to fabricate scaffolds out of other materials such as collagen to see if this method is widely applicable.

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