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Fabrication and release behavior of a novel freeze-gelled chitosan/γ-PGA scaffold as a carrier for rhBMP-2

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Summary *Objective*: The aim of this study was to fabricate a novel composite porous scaffold by blending chitosan and gamma-poly(glutamic acid) (γ-PGA) for the sustained delivery of rhBMP-2.
Methods: Chitosan and γ-PGA were blended to fabricate a novel porous scaffold by the freeze-gelation method. For comparison, scaffolds made of freeze-dried chitosan, freeze-dried PLLA, and freeze-gelled chitosan were also prepared. The scaffolds were loaded with rhBMP-2, and then the controlled release of rhBMP-2 from the scaffolds was assessed by ELISA.
Results: The freeze-gelled chitosan/γ-PGA scaffold ($M_0=318.29$ ng, $k=0.32\ d^{-1}$) gave the most satisfactory release curve, followed by the freeze-gelled chitosan ($M_0=392.76$ ng, $k=0.59\ d^{-1}$), freeze-dried chitosan ($M_0=229.21$ ng, $k=2.28\ d^{-1}$), and freeze-dried PLLA ($M_0=8.4$ ng, $k=482.54\ d^{-1}$) scaffolds. In the stability test, *p*-dioxane (the solvent for PLLA) seriously deteriorated rhBMP-2, whereas acetic acid (the solvent for chitosan) did not.
Significance: A novel chitosan/γ-PGA composite scaffold for the controlled release of rhBMP-2 was established, with an enhanced release amount and sustained release behavior. This scaffold has many potential applications in bone regenerative therapies.

Introduction

The implantation of functional materials into the human body to replace original tissues has been carried out for decades. Tissue engineering provides a novel way to recover physiological function

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by seeding cells onto scaffolds, together with the use of growth factors to modulate cell proliferation [1,2]. The function of a tissue can thus be restored when the cells on the scaffold organize into a mature tissue. Hence, integration among scaffold materials, cells, and signaling molecules is becoming critically essential in the engineering of regenerated tissue. In many cases where growth factors play an important role, a sufficient amount of, and a satisfactory release profile for the growth factor, to competently meet the needs of the proliferating cell mass, are required [1,3–8]. Taking applications to bone defects as an example, the use of biomaterials containing bone morphogenetic proteins (BMPs) at the defect significantly accelerates the healing process [9].

BMPs are often found in bone, cartilage, and other tissues such as osteosarcoma tissue, nerve tissue, etc. BMPs are low molecular weight glycoproteins, which are members of the transforming growth factor-(β (TGF-β) superfamily [9,10]. BMPs have been known to promote the morphogenesis of bone and cartilage tissues by modulating and inducing the growth of osteoblasts and chondroblasts [11–13]. Studies have shown that the duration of obvious ectopic bone formation induced by BMP-2 is about 2 weeks [14]. The direct injection of BMPs usually cannot result in the desired osteogenic effect because of in vivo metabolism and enzymatic digestion, which result in a very short half-life. In order to provide the stable, sustained release of BMPs and also a three-dimensional growth scaffold for cells, a proper carrier for BMPs is required [15]. There are two important characteristics of the sustained-release profile of an embedded protein factor: the total release amount (concentration) at each time point and the effective release duration. According to the postulation mentioned above [14], a carrier scaffold, which has a large total release amount and long effective release duration, is most suitable for the delivery of BMPs

Several kinds of natural and synthetic carriers have commonly been used for the delivery of BMPs in tissue engineering, including collagen [16], poly(DL-lactide-co-glycolide) (PLGA) [17–19], poly(L-lactic acid) (PLLA) [20–23], alginate [24], synthetic hydroxyapatite [25], etc. Carriers made of collagen and alginate lack good mechanical strength; carriers made of PLLA and PLGA, although sufficiently strong, have poor hydrophilicity, and traces of toxic solvent used in manufacturing the scaffold may pose a problem [26]; while carriers made of hydroxyapatite cannot effectively carry growth factors.

Chitosan, constituted by glucosamine and *N*-acetylglucosamine, is a polysaccharide derived from the *N*-deacetylation of chitin [27,28]. Chitosan is positively charged in acidic and neutral solutions. Chitosan can be degraded by lysozymes in vivo, leading to the release of a biocompatible product, glycosaminoglycan. The degradation rate of chitosan is relatively slow and is influenced by the degree of deacetylation. Chitosan has been widely used in various biomedical applications [28–35]. Gamma-poly(glutamic acid) (γ-PGA) is a poly-amino acid formed by the amide bond linkage between the amino group on the α-carbon and the carboxyl group on the γ-carbon. γ-PGA has fairly high viscosity, adequate hydrophilicity, and is biodegradable. Thus, γ-PGA has been successfully utilized in bioglue and drug delivery systems [36–42]. γ-PGA is negatively charged in aqueous acids and thus can produce ionic cross-linking with chitosan.

Based on the characteristics of chitosan and γ-PGA, a method in the present study was developed to fabricate a porous chitosan/γ-PGA composite scaffold. The use of chitosan and γ-PGA was proposed to fabricate a novel scaffold as a carrier system to deliver BMP-2 and characterize the release behavior of BMP-2 embedded in this scaffold. For comparison, four types of scaffold were prepared using freeze-gelation or freeze-drying methods. These scaffolds. including freeze-gelled chitosan/γ-PGA, freeze-gelled chitosan, freeze-dried chitosan, and freeze-dried PLLA, were loaded with rhBMP-2 and then subjected to an in vitro controlled-release assay. The solvents used in the fabrication processes of these scaffolds may also affect the stability of rhBMP-2's activity. Therefore, the effect of the solvents on the stability of rhBMP-2 was also investigated.

Materials and methods

Materials

Chitosan was purchased from the Taiwan Chitin Chitosan Co. (Taipei, Taiwan) (with a M_w of 400,000 and degree of deacetylation of 90%). Gamma-poly(glutamic acid) was kindly provided by Prof. Yao-Nan Chang, Department of Bioindustry Technology, Da-Yeh University, Changhua, Taiwan. PLLA (poly(L-latide)) was obtained from Fluka (Buchs, Switzerland). Recombinant human bone morphogenetic protein 2 (rhBMP-2) and the ELISA kit for rhBMP-2 were purchased from R&D Systems (Minneapolis, MN, USA). Acetic acid, *p*-dioxane, ethanol, and sodium hydroxide were obtained from J.T.

Baker (Philipsburg, NJ, USA). Phenylmethanesulfonyl fluoride (PMSF), phosphate-buffered saline (PBS) tablets, sodium azide, and sodium fluoride were purchased from Sigma or Sigma–Aldrich (St Louis, MO, USA).

Preparation of the freeze-gelled chitosan scaffolds and freeze-gelled chitosan/γ-PGA scaffolds

The freeze-gelation method was chosen to fabricate the scaffolds [26]. In this method, the frozen scaffold solution was immersed in a gelation solution at a temperature lower than its freezing point. The scaffold had already gelled before the drying stage, thus the porous structure could also be retained without lyophilization. The advantages of the freeze-gelation method are time and energy savings, low residual solvent content, and easy scale-up [26]. In this study, a chitosan solution (4 wt%) and chitosan/γ-PGA solution (chitosan 4 wt%, γ-PGA 1 wt%) were prepared by placing chitosan and γ-PGA in 0.2 M acetic acid, stirring at 750 rpm for 12 h, then centrifuging for 15 min at $6500 \times g$. Eighty microliters of the solution were then poured into dishes with an inner diameter of 8 mm and frozen for 12 h with the temperature maintained at −80 °C. The frozen samples from each preparation were then immersed in a 3 M NaOH/ethanol solution at −20 °C for 12 h, followed by rinsing with ethanol and PBS solution.

Preparation of the freeze-dried chitosan scaffolds and freeze-dried PLLA scaffolds

A chitosan solution (4 wt%) was prepared using the same procedure as described in Section 2.2, and a PLLA solution (5 wt%) was prepared by dissolving PLLA pellets in *p*-dioxane. Eighty microliters of the solution were then poured into dishes with an inner diameter of 8 mm and frozen for 12 h at a temperature of −80 °C. The frozen samples were lyophilized in a freeze-dryer (Heto, model LyoLab 3000, Allerod, Denmark) for 4 days.

Analysis of the scaffold structure by SEM

The structures of the scaffolds were examined by scanning electron microscopy (SEM). Scaffold samples were rinsed in a 95% ethanol solution at −20 °C for 12 h and then dried in a lyophilizer. The samples were coated with gold–palladium and then examined by SEM.

Loading of rhBMP-2 on scaffolds

In the present study, rhBMP-2 was loaded into scaffolds using two methods. For scaffolds prepared by the freeze-gelation method, the rhBMP-2 solution was dropped into the scaffolds. Then the scaffolds were dried at 4 °C for 24 h. For freeze-dried scaffolds, the rhBMP-2 solution was directly added into the solution of chitosan or PLLA before lyophilization. The amount of rhBMP-2 loaded into each scaffold was 1000 ng.

Controlled release of rhBMP-2 from scaffolds

To investigate the release kinetics of rhBMP-2 from scaffolds, the ELISA kit was utilized for detecting rhBMP-2 in the experimental media. To prevent contamination by bacteria and rapid degradation of rhBMP-2 in the buffer, PMSF (0.4 mM), sodium fluoride (2 mM), and sodium azide (10 mM) were added as a protease inhibitor and germicide. Scaffolds were put into tubes containing 5 ml PBS at 37 °C, with shaking at 100 rpm in an orbital shaker. The buffer samples were taken at the time points of 0, 6, 12, and 24 h and 4, 7, 10, and 14 d and assayed for rhBMP-2.

Analysis of the release behavior of rhBMP-2

As mentioned above, an ELISA kit was utilized to determine the concentration of rhBMP-2 in the in vitro controlled-release experiment. The traditional in vivo observation of osteogenesis can only indicate the presence of rhBMPs, but the detailed release profile cannot be obtained. In contrast, these experiments were able to provide quantitative information about the release amount and release rate of rhBMP-2. Furthermore, the authors derived a mathematical model in the following sections to analyze the release mechanism of rhBMP-2 from various scaffold formulations.

According to the mass transfer theory, one can apply the equation of change for mass transfer to demonstrate that the relationship between concentration and space varies with time [43]

$$\frac{\partial C}{\partial t} + \vec{v} \cdot \nabla C = D \cdot \nabla^2 C + R; \quad (1)$$

where C is the concentration of rhBMP-2, t is the time, v is the velocity of the fluid, D is the mass transfer coefficient, and R is the change of concentration caused by the chemical reaction.

The scaffold in this experiment was a thin disc; therefore cylindrical coordinates were applied to integrate the volume of the disc, with radius, R,

and height represented by L (the Z-axis). Since the scaffold was circularly symmetrical, $\partial C/\partial\theta = 0$. In comparison with the area of bottom and surface, the area of the lateral side of the disc was rather small, so the mass transfer flux through the lateral side could be neglected ($\partial C/\partial r = 0$). Assuming that no chemical reaction occurs and the concentration of rhBMP-2 released in the buffer is nearly zero (if the volume of buffer is much greater than the volume of the disc), then the equation of change for rhBMP-2 can be simplified as follows:

$$\frac{\partial C}{\partial t} = D\frac{\partial^2 C}{\partial z^2}. \tag{2}$$

The initial and boundary conditions are, $t = 0$, $C = C_0$; $Z = 0$, $C = 0$; and $Z = L$, $C = 0$, respectively. The solution of Eq. (2) is [44]:

$$C = C_0 \sum_{n=0}^{\infty} \frac{-4}{(2n+1)\pi} \sin\left[\frac{(2n+1)\pi z}{L}\right] \times \exp\left[\frac{-(2n+1)^2\pi^2 D}{L^2} t\right]. \tag{3}$$

Integrating Eq. (3) with respect to volume, one can obtain the residual amount (M) of rhBMP-2 in the scaffold at time t

$$M = C_0\pi R^2 L \times \sum_{n=0}^{\infty} \frac{8}{(2n+1)^2\pi^2} \exp\left[\frac{-(2n+1)^2\pi^2 D}{L^2} t\right]; \tag{4}$$

where $C_0\pi R_2 L$ is M_0, the initial amount of rhBMP-2, which is equivalent to the equilibrium release amount. Making an approximation by using the first term of the infinite series, the previous equation (Eq. (4)) can be simplified to $M = M_0\, e^{-kt}$, where k is the time constant. Thus, the amount of rhBMP-2 released in the buffer can be written as $M_{released} = M_0 - M = M_0(1 - e^{-kt})$. Using this equation to fit the curves with experimental data from the present study, the constants M_0 and k can be obtained. A large M_0 indicates that a large amount of rhBMP-2 is available for release from the scaffold. A small k indicates that slow but sustained release of rhBMP-2 can be expected. By using the values of M_0 and k, the release behavior of different carrier scaffolds for rhBMP-2 were characterized.

Stability test of rhBMP-2 in various solvents

To investigate the stability of rhBMP-2 in different solvents, rhBMP-2 was put into 0.2 M acetic acid, 50% *p*-dioxane, or PBS. PMSF, sodium fluoride, and sodium azide were added as a protease inhibitor and germicide. To ensure the efficacy of the additives, a blank sample without additives was taken as the control. rhBMP-2 (1000 ng) was added to 5 ml of solvent in a centrifuge tube at 37 °C, with shaking at 100 rpm in an orbital shaker. Buffer samples were taken at the time points of 0, 6, 12, and 24 h and 4, 7, 10, and 14 d and then subjected to the rhBMP-2 assay.

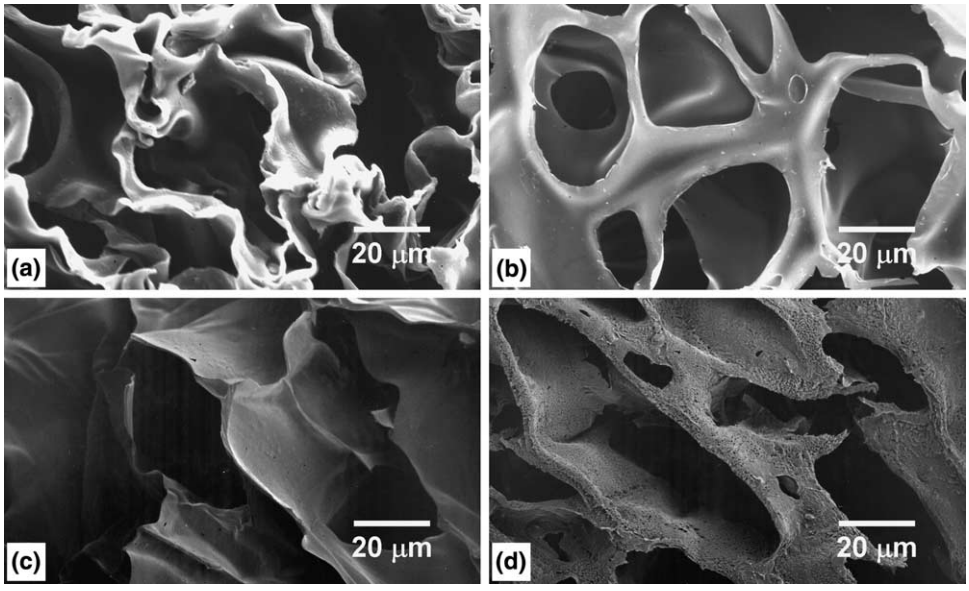

Figure. 1 SEM micrographs of cross-sections of (a) freeze-gelled chitosan, (b) freeze-gelled chitosan/γ-PGA, (c) freeze-dried chitosan, and (d) freeze-dried PLLA scaffolds.

Table 1 Pore sizes of different scaffolds.

Scaffold	Freeze-gelled chitosan	Freeze-gelled chitosan/γ-PGA	Freeze-dried chitosan	Freeze-dried PLLA
Pore size (μm)	33.9 (10.4)*	20.7 (3.8)*	53.9 (12.7)**	66.5 (17.8)

Values are the means (SD), $n=10$. *$p<0.005$ compared with freeze-dried PLLA. **$p<0.5$ compared with freeze-dried PLLA.

Results

SEM analysis for porous scaffolds

The structure of scaffolds was investigated by SEM (Fig. 1). According to the SEM micrographs, all four types of scaffold were found to be porous. It should be noted that unlike the other three scaffolds, there were many tiny pores on the surface of the freeze-dried PLLA scaffolds (Fig. 1d). Furthermore, the pore sizes of these scaffolds were also determined from the SEM micrographs. The data indicates that the scaffolds made by the freeze-gelation method had smaller pore sizes than those made by the freeze-drying method (Table 1).

Controlled release of rhBMP-2 from scaffolds

The release profiles of rhBMP-2 from the four different scaffolds are displayed in Fig. 2. The equation $M_{released} = M_0(1-e^{-kt})$ was used to fit the experimental data given in Fig. 2, and the obtained parameters are listed in Table 2. These parameters were used to plot the equation curves, as shown in Fig. 2. Parameter M_0 can be regarded as the release amount at equilibrium, k is the time constant, for which a higher value indicates a faster release rate. By using values of M_0 and k, one can calculate the time needed for $M_{released}$ to reach 50 and 75% of M_0 (Table 2, $T_{50\%}$ and $T_{75\%}$); one can also calculate the percentage of rhBMP-2 released on days 1 and 2 (Table 2, M_{day1} and M_{day2}). Clearly, when each scaffold was loaded with the same amount of rhBMP-2, the scaffolds fabricated by the freeze-gelation method showed higher M_0 values (equilibrium release amount) than those fabricated by the freeze-drying method.

The magnitude of the k value is an index of the release rate. The release rates of rhBMP-2 from the freeze-dried chitosan and especially PLLA scaffolds were strikingly fast (Table 2, $T_{50\%}$ and $T_{75\%}$); more than 75% of rhBMP-2 was released on day 1. (Only the rate of release percentage was evaluated here. The total amount of BMP released from the PLLA was very small. The reason is discussed in Section 3.3.) For the freeze-gelled chitosan, it took 2.36 days to release 75% of the rhBMP-2. In contrast, the release of rhBMP-2 from the freeze-gelled chitosan/γ-PGA scaffolds were relatively slow; it took 2.18 and 4.36 days to release 50 and 75% of the rhBMP-2, respectively. Similar trends were seen in the M_{day1} and M_{day2} values, which indicate a long-lasting release of rhBMP-2 if these values are relatively low (Table 2). Based on these results, the freeze-gelled chitosan/γ-PGA scaffolds are recommended as carriers for rhBMP-2 because they release relatively higher amounts of rhBMP-2 in a more sustained manner, thus they are expected to potentially induce effective bone morphogenesis.

Stability test of rhBMP-2 in solvents

In this study, two different solvents were used to prepare the scaffolds: an acetic acid solution was the solvent for chitosan and γ-PGA, and dioxane was the solvent for PLLA. The presence of these solvents may decrease the stability of rhBMP-2's activity and thus significantly affect its efficacy. To investigate this effect, variations of the residual rhBMP-2 concentration with respect to time in four different solutions are shown in Fig. 3. The acetic acid solution (0.2 M) and 50% dioxane were the solvents for chitosan/γ-PGA and PLLA, respectively. PBS was the buffer used for the in vitro controlled-release experiment described in Fig. 2.

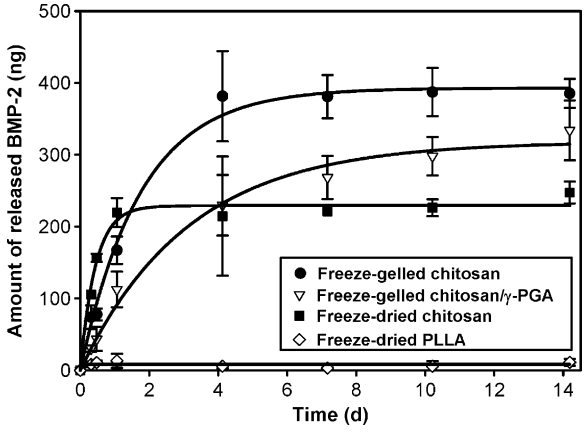

Figure 2 Release of BMP-2 from different scaffolds. BMP-2 content, 1000 ng/scaffold. The curves were derived from fitting the data using the equation $M_{released} = M_0(1-e^{-kt})$. See Section 2.7 for more details.

Table 2 Parameters for the release of rhBMP-2.

Scaffold	M_0 (ng)	K (d^{-1})	$T_{50\%}$[a] (d)	$T_{75\%}$[a] (d)	M_{day1}[b] (%)	M_{day2}[b] (%)
Freeze-gelled chitosan (•)	392.76	0.59	1.18	2.36	43.39	67.95
Freeze-gelled chitosan/γ-PGA (▽)	318.29	0.32	2.18	4.36	27.22	47.04
Freeze-dried chitosan (■)	229.21	2.28	0.3	0.61	89.74	98.95
Freeze-dried PLLA (◇)	8.4	482.54	0.0014	0.0029	100	100

These values were obtained by curve fitting the data in Fig. 2 using the equation $M_{released} = M_0(1-e^{-kt})$.
[a] $T_{50\%}$ and $T_{75\%}$, the time needed for $M_{released}$ to reach 50 and 75% of M_0, respectively.
[b] M_{day1} and M_{day2}, the percentage of BMP-2 released on days 1 and 2, respectively.

To prevent degradation of rhBMP-2 in different solutions, PMSF, sodium fluoride, and sodium azide were added to the PBS as a protease inhibitor and germicide. To ensure the efficacy of these additives in inhibiting rhBMP degradation, one blank sample (PBS without additive) was taken as the control. From the results shown in Fig. 3, rhBMP-2 was found to be seriously deteriorated in dioxane, and only 5% of rhBMP-2 remained detectable in the solvent after 4 days. In contrast, rhBMP-2 was more stable in the acetic acid solution; 20% of the rhBMP-2 could still be detected after 2 weeks even in the acetic acid solution.

The necessity of the additives (PMSF, sodium fluoride, and sodium azide) was also demonstrated in Fig. 3. rhBMP-2 appeared to be rapidly degraded in PBS without additives. As a comparison, rhBMP-2 remained relatively stable in PBS buffer with additives.

Discussion

In this study, chitosan and γ-PGA were blended to fabricate a novel composite scaffold intended to be used as a new rhBMP-2 carrier. To prevent residual organic solvent remaining and the associated deterioration of rhBMP-2, a freeze-gelation method, instead of a freeze-drying method, was used. From the data in the present study, the amount of rhBMP-2 released in the PBS solution from scaffolds made by the freeze-gelation method was noted to be higher than that of scaffolds made by the freeze-drying method. This difference could be attributed to the different manufacturing processes. In the freeze-drying method, rhBMP-2 was directly added into the acetic acid or dioxane solution for preparing scaffolds, which was then dried after being frozen at −80 °C, so the rhBMP-2 may have been partially damaged either by the solvent or by the low-temperature drying process. On the other hand, rhBMP-2 was loaded after the formation of scaffolds when using the freeze-gelation method, and thus the rhBMP-2 had much less chance to be damaged, leading to a higher release amount (M_0).

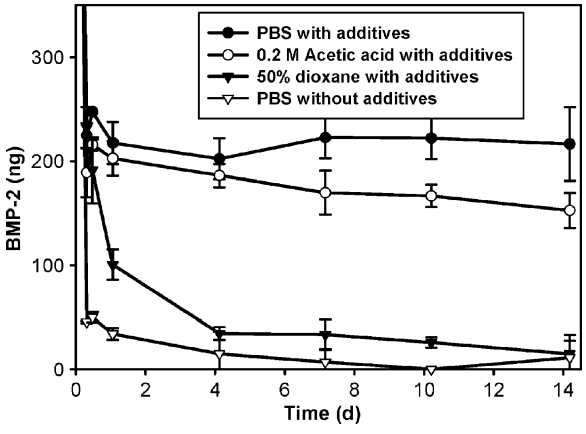

Figure 3 Stability of BMP-2 in various solutions. BMP-2 content, 1000 ng/5 ml.

The release amount of rhBMP-2 was dramatically lower for the freeze-dried PLLA scaffolds than for the other scaffolds (Fig. 2, Table 2). Consequently, together with the data illustrated in Fig. 3, it can be inferred that rhBMP-2 was seriously deteriorated by dioxane during the manufacturing process, and therefore, less rhBMP-2 was released. When rhBMP-2 was dissolved in 50% dioxane, <5% of the loaded amount was detected after 4 days, suggesting that most of the rhBMP-2 had been destroyed in the preparation process. Nevertheless, since a minute amount of rhBMP-2 can be effective in vivo, many studies claim that PLLA is a suitable carrier for rhBMP-2 based on the histological data [20–23]. Considering the fact that rhBMP-2 is rather expensive, the fabrication process of PLLA scaffolds should be improved to enhance the utilization of rhBMP-2. Unlike in dioxane, the concentration of rhBMP-2 remained constant in the acetic acid solution during a 14-day period (Fig. 3) in this study. Thus, it was concluded that rhBMP-2 was relatively stable in the acetic acid solution.

The release rate of rhBMP-2 from the freeze-dried chitosan scaffolds in the early phase (2–3 days) was found to be faster than that from the freeze-

gelled chitosan scaffolds (Fig. 2, Table 2). According to the pore size analysis (Table 1), it was found that scaffolds made by the freeze-gelation method had smaller pores and a larger mass transfer resistance, and thus a slower release rate of rhBMP-2 was obtained. On the other hand, the chitosan/γ-PGA scaffolds had a slower release rate in the early phase (2–3 days) than the other two chitosan scaffolds. Compared with chitosan, γ-PGA is a poly-amino acid with many properties similar to proteins. Thus, γ-PGA may have a better affinity to rhBMP-2, and explains why the chitosan/γ-PGA scaffolds displayed a slow, sustained release behavior for rhBMP-2 (Fig. 2). rhBMP-2 induces the formation of bones, and apparent osteogenesis can be observed in approximately 2 weeks [45]. Since rhBMP-2 is vulnerable when dissolved in vivo, an ideal carrier is needed with characteristics that can protect and maintain the release of rhBMP-2 for a certain period of time. Among the four different scaffolds used in this study, the freeze-gelled chitosan scaffolds had the highest equilibrium release amount (M_0). But the release rate was slightly too fast: almost 75% of the rhBMP-2 had been released after 2 days. The freeze-gelled chitosan/γ-PGA scaffolds seemed to have a slightly lower equilibrium release amount than the freeze-gelled chitosan scaffolds, whereas they exhibited a better (comparatively slower and more-sustained) release rate.

The amounts of rhBMP-2 (~200 ng) in the PBS- and acetic acid-antiprotease solution were lower than those determined in the chitosan-based scaffolds (200–400 ng). This may indicate that part of the rhBMP-2 is trapped in the scaffolds (especially the freeze-gelled ones) and is yet to be released. This data supports the fact that the scaffolds made by the freeze-gelation method can successfully protect rhBMP-2. The effect may be ascribed to the steric hindrance provided by the scaffolds, thus preventing protease binding to the trapped rhBMP-2.

In this study, the release behavior of rhBMP-2 in a PBS solution was determined using an ELISA kit instead of using observations of in vivo osteogenesis. The reason is that only a small, effective amount of rhBMP-2 is sufficient to induce the generation of bone and cartilage tissues in vivo, but the accurate amount of rhBMP-2 released from scaffolds in previous studies was not determined [20,22,23]. Because of its relatively short half-life and high price, finding a way to efficiently deliver rhBMP-2 is very important in the clinical applications for tissue engineering. Although some studies have indicated that PLLA is a suitable carrier for rhBMP-2 [20,22,23], based on this data, the authors suggest that the novel freeze-gelled chitosan/γ-PGA scaffold seems to be the best one among the four types of scaffolds tested in the present study, since it gave a reasonably higher initial release and also more-sustained release behavior later on.

Conclusions

This novel freeze-gelled chitosan/γ-PGA scaffold has been developed for a reasonably sustained and large amount of release of rhBMP-2. rhBMP-2, which is also relatively stable in an acetic acid solution, is the solvent used to fabricate this scaffold. These results indicated that this novel scaffold is very promising as a carrier system for rhBMP-2 and that this novel biomaterial can be further developed and potentially applied to the therapy of bone defects and other related diseases.

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