Novel Functional Biodegradable Polymer IV: pH-Sensitive Controlled Release of Fibroblast Growth Factor-2 from a Poly(γ-glutamic acid)-Sulfonate Matrix for Tissue Engineering

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The acidic pH-sensitive controlled release of fibroblast growth factor-2 (FGF-2) from a biodegradable hydrogel without any denaturation of the FGF-2 was successfully performed by a combination of FGF-2 activity and acidic pH-sensitivity. We prepared semi-interpenetrating polymer network like hetero-gels (S72-netgels) composed of poly(γ-glutamic acid) (γ-PGA) and 72% sulfonated γ-PGA (γ-PGA-S72). S72-netgels including 36 mol % sulfonic acid (S72-netgel-36) showed wide acidic pH-sensitive deswelling properties at pH = 2.0−6.0, corresponding to the isoelectric point of carboxylic acid, because of the concentration of protons due to the neighboring sulfonic acids from the carboxylic acids. The S72-netgel-36 (the volume of hydrogel is 7.85×10^{-2} cm^3) can incorporate 280 ng of FGF-2 after 24 h immersion in Tris-HCl buffer (pH = 7.4), including 1.0 μg of FGF-2. The S72-netgel-36 still retained about 60% of the FGF-2 even after 15 days of incubation in fresh Tris-HCl buffer at 37 °C because of the stable interaction of FGF-2 with γ-PGA-S72 in S72-netgel-36. The release of FGF-2 from the S72-netgel-36 was successfully controlled by alternating immersion in pH = 7.4 and acidic pH buffers. Furthermore, the FGF-2 released from the S72-netgel-36 retained its activity without denaturation because the γ-PGA-S72 in S72-netgel-36 has a protective activity. The acidic pH-sensitive FGF-2 release property of the S72-netgel-36 without denaturation of the FGF-2 may be useful for tissue engineering fields such as neovascular treatment for ischemia and inflammation.

Introduction

The injection of growth factor solutions has been used to heal and regenerate tissues and organs. However, it is difficult to directly apply bioactive proteins to tissue-engineering processes because of their sensitivity to denaturation and degradation by external environmental factors such as pH, organic solvents, sonication, and enzymes.[1,2] Many trials have been performed to control the release of growth factors using synthetic polymer hydrogels and naturally occurring polymer hydrogels.[3−7] However, the localized and stimulus-sensitive controlled release of growth factors from conventional hydrogels has not been achieved yet due to the primary burst and deactivation of the released growth factors. Furthermore, the use of excessive amounts of growth factors carries a high risk, which may trigger the growth of cancer cells. To develop the localized and stimulus-sensitive controlled delivery of growth factors from hydrogels, we have proposed the design and preparation of biomaterials with growth-factor activity and stimulus sensitivity. It is therefore desirable to develop hydrogels with growth factor activity that can stably interact with the hydrogel over a long time and for the activity of the released growth factors to be maintained because the hydrogel can protect them from environmental denaturation like proteoglycans such as heparansulfate proteoglycan. Furthermore, a strategy for the stimulus-sensitive delivery of growth factors to a target area is required for localized and controlled release.

In previous studies, we reported the design, synthesis, and fibroblast growth factor-2 (FGF-2) activities of sulfonated poly(γ-glutamic acid) (γ-PGA-S) and its hydrogels.[8−10] γ-PGA is a naturally occurring polymer secreted by a *Bacillus subtilis* strain and provides a potential resource for environmental and biodegradable materials.[11−13] 72% sulfonated γ-PGA-S (γ-PGA-S72) has higher FGF-2 mitogenic and protective activities than heparin or heparinoids, because it has the appropriate molar ratio of carboxyl and sulfonic groups for electrostatic interaction with the cationic amino acids of the FGF-2 binding site.[9]

In this study, we report a novel strategy to develop the acidic pH-sensitive controlled delivery of FGF-2 without denaturation from γ-PGA-S hydrogels to the local acidic pH found in various diseased states such as inflammation and ischemia. The delivery of FGF-2 to an ischemic area has been studied and is desirable for neovascular treatment. For example, Marui et al. reported that FGF-2 and hepatocyte growth factor delivery from collagen microspheres enhanced blood vessel formation in the murine ischemic hindlimb.[14] Yajima et al. reported that the injection of FGF-2 and heparin solution suppressed ischemic heart disease.[15] Laham et al. concluded that FGF-2 delivery from heparin-alginate microcapsules promoted the growth of new blood vessels in

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Table 1. Preparation of the γ-PGA Hydrogel and S72-netgels[a]

	sulfonic acid mol %[b] (%)	γ-PGA-S72 (unit mmol)	γ-PGA (unit mmol)	γ-PGA-S72/ γ-PGA	EGDGE[c] (mmol)	[epoxy]/ [COOH][d]	swelling ratio[e]
γ-PGA gel	0		10		10		68
S72-netgel-6.5	6.5	0.9	9.0	0.1	9.0	2.0	71
S72-netgel-17	17	2.3	7.7	0.3	7.7		70
S72-netgel-36	36	5.0	5.0	1.0	5.0		77

[a] Reaction time was 48 h, reaction temperature was 40 °C, pH = 4.5. [b] The sulfonic acid molar percentages were calculated by following equation. Sulfonic acid molar percentage (%) = {unit mmol of γ-PGA-S72/10 unit mmol (total mmol of γ-PGA-S72 and γ-PGA)} x 0.72. (sulfonic acid molar ratio in γ-PGA-S72) × 100. [c] Ethyleneglycol diglycidyl ether (EGDGE) is cross-linking agent for γ-PGA. [d] Carboxyl group of γ-PGA. [e] Swelling ratio = (W_s - W_d)/W_d, W_s and W_d were the weight of the swelling and dry hydrogels, respectively. Polymer concentrations were 20 wt %.

the ischemic myocardium.[16] Tabata and co-workers studied FGF-2 release from gelatin hydrogels in order to induce angiogenesis and collateral vessels for ischemia.[5,17] However, the FGF-2 release behavior was not controllable by their gelatin hydrogel system because the FGF-2 release depended on the degradation of the gelatin hydrogel.[18] To develop the stimulus-sensitive controlled delivery of FGF-2 to an ischemic disease, we observed the relationship between the pH and the ischemic area. It has been reported that the ischemic and inflamed areas in the body have a lower pH (<6.5) than the surrounding tissues and blood (pH = 7.4).[19,20] If a hydrogel can release FGF-2 without denaturation by recognizing the environmental pH and then stop the release by altering the environmental pH from acidic to neutral, this hydrogel might be useful as an intelligent-controlled release material. In this report, we evaluated that pH-sensitive deswelling properties of a semi-interpenetrating polymer network (semi-IPN) like hetero-gel consisting of γ-PGA and γ-PGA-S72 (S72-netgel). Furthermore, the acidic pH-sensitive controlled release of FGF-2 from S72-netgels and the activity of FGF-2 released from S72-netgels were evaluated by a mouse L929 fibroblast proliferation study under serum free conditions.

Experimental Section

Materials. All reagents were used as purchased without further purification. γ-PGA (M_w = 310 000, M_w/M_n = 1.20) was kindly donated by Meiji Seika Kaisya, Ltd. (Tokyo, Japan). Sodium chlorate, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (WSC), sodium dextran sulfate (DS; M_w = 500 000), and 2-aminoethanesulfonic acid (taurine) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Recombinant human FGF-2 was purchased from Sigma−Aldrich (Missouri, U.S.A.). Fetal bovine serum (FBS) was purchased from Sigma (St. Louis, MO). WST-1 reagent was purchased from Dojindo Ltd. (Kumamoto, Japan). The ELISA kit Quantikine HS of human FGF basic was purchased from R&D system Inc. (Minneapolis, U.S.A.). All other chemicals were purchased from Nakarai Tesque (Kyoto, Japan).

Synthesis of γ-PGA-S72. 72% sulfonated γ-PGA-S (γ-PGA-S72) was synthesized according to our previous study.[8] Briefly, 645 mg (5.0 unit mmol) of γ-PGA, 1.2 g (78 mmol) of WSC, and 626 mg (5.0 mmol) of taurine were dissolved in 0.5 M aqueous sodium hydrogen carbonate under magnetic stirring for 30 min at 0∼4 °C. The reaction solution was then maintained for 24 h at ambient temperature. The reaction solution was dialyzed using a Spectra/Pore membrane (cutoff molecular weight of 50 000) for 3 days. The γ-PGA-S72 was obtained by freeze-drying the solution for 3 days. The degree of sulfonation of γ-PGA was evaluated by ^{1}H NMR and FT-IR spectra.[8]

Preparation of γ-PGA Hydrogel and S72-Netgels. γ-PGA hydrogel and semi-interpenetrating polymer network (semi-IPN) like hetero-gel consisted of γ-PGA and γ-PGA-S72 (S72-netgel) were prepared according to our former study.[10] The condition of the hydrogels is listed in Table 1. Ten unit mmol γ-PGA was dissolved in 0.5 M aqueous sodium hydrogen carbonate. Ethylene glycol diglycidyl ether (EGDGE) was used as a cross-linker. The epoxy group concentration of EGDGE was 2-fold higher than the carboxyl group concentration of γ-PGA. The pH of the solution was adjusted to 4.5, and the solution was maintained at 40 °C for 48 h. The remaining compounds in the hydrogels were removed by soaking the hydrogels in pure water for 3 days. The S72-netgels were prepared as follows: various amounts of γ-PGA and γ-PGA-S72 were dissolved in 0.5 M aqueous sodium hydrogen carbonate. An adequate amount of EGDGE was then added to the reaction solution. The polymer solution was kept at 40 °C for 48 h, at a pH of 4.5. Residual compounds in S72-netgels were removed by pure water dialysis for 3 days. We used disk type hydrogels (the diameter is 1 cm, the height is 1 mm, and the volume is 7.85×10^{-2} cm^3) for the evaluations of swelling and FGF-2 release properties.

Time Dependence Swelling Ratio of γ-PGA Hydrogel and S72-Netgels in Acidic Solution. γ-PGA and various S72-netgels were immersed in 0.01 N NaCl aqueous solution (pH = 7.4) for 24 h at 25 °C, and then their diameters were observed by a Stemi DV4 microscope (Carl Zeiss, German) connected to a Cybershot DSC-F505V digital camera (Sony, Japan). The hydrogels were immersed in 0.01 N acidic solution (pH = 2.0) for 16 h, and the diameters were measured after the prescribed times. The swelling ratios were calculated from the ratio between the diameter at the corresponding time (D) and the diameter of the hydrogels in 0.01 N NaCl aqueous solution (pH = 7.4) (D_0): D/D_0.

pH Dependence Swelling Ratio of γ-PGA Hydrogel and S72-Netgels. γ-PGA and the various S72-netgels were immersed in 0.01N NaCl aqueous solution (pH = 7.4) for 24 h at 25 °C, and then their diameters were observed by a Stemi DV4 microscope (Carl Zeiss, German) connected to a Cybershot DSC-F505V digital camera (Sony, Japan). The

hydrogels were immersed in 0.01 N acidic solutions (pH = 2.0, 2.5, 3.0, 4.0, and 6.0) for 24 h at 25 °C, and the diameters of equilibrium-swelling hydrogels were measured. The pH dependence swelling ratios were calculated from the ratio between the diameter of equilibrium-swelling hydrogels in each acidic solution (D) and the diameter of the hydrogels in 0.01 N NaCl aqueous solution (pH = 7.4) (D_0): D/D_0.

Deswelling Kinetics of γ-PGA Hydrogel and S72-Netgels. To analyze the deswelling process quantitatively, we applied a semilogarithmic plot as a first-order rate analysis to the time dependence of the deswelling as follows:

$$\ln[(D - D_\infty)/(D_0 - D_\infty)] = -kt$$

where D_∞ is the diameter of the equilibrium-swelling hydrogels in 0.01 N pH = 2.0 aqueous solution, k is a constant, and t is time. The larger the slope of a plot of $\ln[(D - D_\infty)/(D_0 - D_\infty)]$ against t was, the faster the deswelling process was.

FGF-2 Releasing Properties from γ-PGA Hydrogel and S72-Netgel-36. γ-PGA hydrogels and S72-netgels including 36 mol % sulfonic acid (S72-netgel-36) were immersed in 5 mL of 10 mM Tris-HCl buffer (pH = 7.4) containing 1.0 μg of FGF-2 for 24 h at 4 °C. After 24 h, the hydrogels were rinsed with 10 mM Tris-HCl twice. The hydrogels were then immersed and incubated in 10 mL of fresh 10 mM Tris-HCl buffer (pH = 7.4) for 15 days at 37 °C. After the prescribed times, 100 μL of the supernatant was collected, and the amount of FGF-2 released from hydrogels was measured by the ELISA method.

pH-Sensitive Controlled Release of FGF-2 from γ-PGA Hydrogel and S72-Netgel-36. γ-PGA hydrogels and S72-netgel-36 were immersed in 5 mL of 10 mM Tris-HCl buffer (pH = 7.4) containing 1.0 μg of FGF-2 for 24 h at 4 °C. After 24 h, the hydrogels were rinsed with 10 mM Tris-HCl twice. The hydrogels were then immersed and incubated in 10 mL of fresh 10 mM Tris-HCl buffer (pH = 7.4) for 24 h at 37 °C. After the incubation, the hydrogels were rinsed with fresh 10 mM Tris-HCl (pH = 7.4) and then the hydrogels were immersed in 10 mL of 10 mM Tris-HCl buffer (pH = 4.0) for 1 h at 37 °C. After the immersion in Tris-HCl buffer (pH = 4.0), the hydrogels were rinsed with fresh 10 mM Tris-HCl buffer (pH = 4.0). We repeated these steps three times. In the case of the pH = 6.0 condition, we carried out the same procedure. After the prescribed times, 100 μL of the supernatant was collected and the amount of FGF-2 released from hydrogels was measured by the ELISA method.

Evaluation of FGF-2 Activity Released from γ-PGA Hydrogel and S72-Netgel-36. The mitogenic activity of the FGF-2 which was released from the hydrogels after 24 h (pH = 7.4) and 1 h (pH = 4.0) of incubation was evaluated by mouse L929 fibroblast cell culture in serum-free Eagle's MEM medium for 96 h. γ-PGA hydrogels and S72-netgel-36 were immersed in 5 mL of 10 mM Tris-HCl buffer (pH = 7.4) containing 1.0 μg of FGF-2 for 24 h at 4 °C. After 24 h, the hydrogels were rinsed with 10 mM Tris-HCl twice. The hydrogels were then immersed and incubated in 10 mL of fresh 10 mM Tris-HCl buffer (pH = 7.4) for 24 h at 37 °C. After the incubation, the hydrogels were rinsed with fresh 10 mM Tris-HCl (pH = 7.4) and then the hydrogels were immersed in 10 mL of 10 mM Tris-HCl buffer (pH = 4.0) for 1 h at 37 °C. After 1 h of incubation, the supernatant was neutralized by NaOH and the amount of FGF-2 in the supernatant was measured by ELISA method. The neutralized supernatant was diluted for preparing 10 ng of FGF-2 by 10 mM Tris-HCl (pH = 7.4). The neutralized Tris-HCl (pH = 7.4) buffer containing 10 ng of FGF-2 was added to pre-adhered mouse L929 fibroblast cells (1.0×10^3/dish) which was incubated for 3 h in serum-free Eagle's MEM medium, and the proliferated cell number after the prescribed time was counted with trypan blue staining on a hemocytometer.

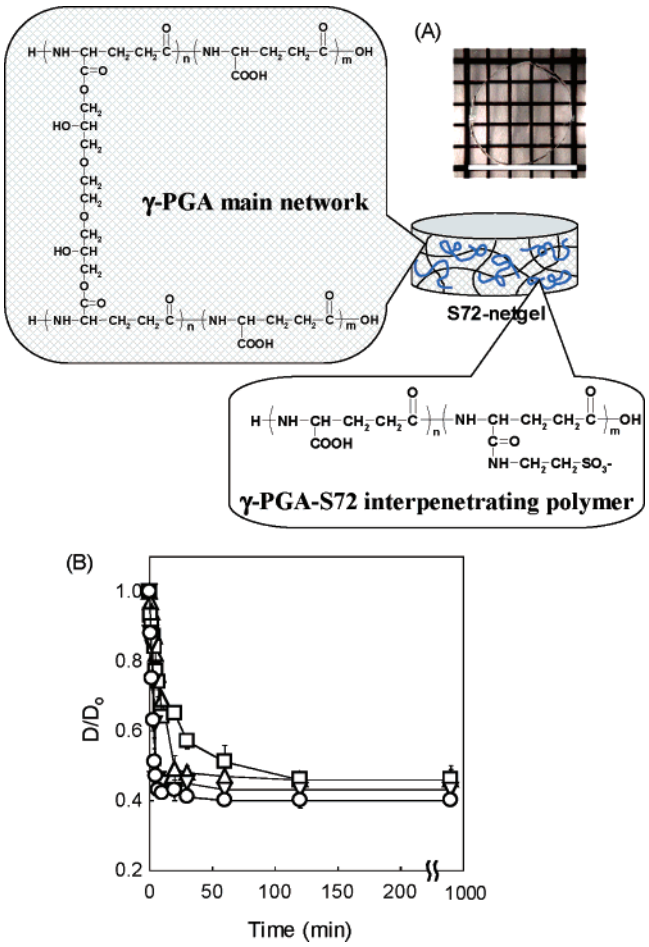

Figure 1. Schematic illustration of a semi-interpenetrating polymer network like hetero-gel (S72-netgels) composed of γ-PGA and γ-PGA-S72 (A). Scale bar is 1 cm. The time dependence swelling ratio of the γ-PGA gel (△) and S72-netgel-6.5 (□), -17 (▽), and -36 (○) in pH = 2.0 aqueous solution for 16 h (n = 3, means ± S. D.) (B). D_0 is the diameter of the hydrogels in 0.01N NaCl aqueous solution (pH = 7.4), and D is the diameter of the hydrogels after immersion in pH = 2.0 solution for the prescribed times.

Results and Discussion

Deswelling and Swelling Properties of γ-PGA Hydrogel and S72-Netgels. Hydrogels with carboxyl groups are known to have deswelling properties corresponding to the acidic pH sensitivity of the isoelectric point of the carboxylic acid.[21−23] We evaluated the pH-sensitive deswelling properties of the γ-PGA hydrogels and S72-netgels in detail. Figure 1 shows the time dependence of the swelling ratio of the

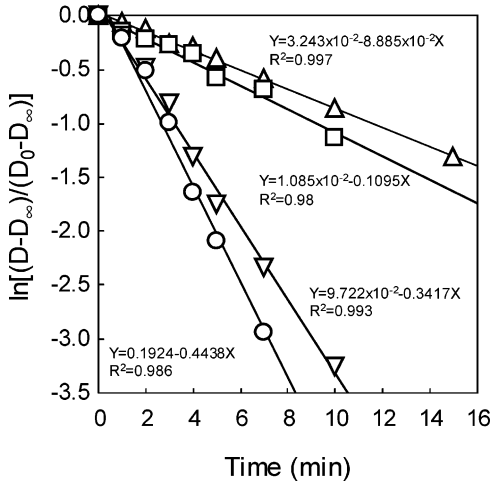

Figure 2. Semilogarithmic plot for the first-order rate analysis of the deswelling process. The γ-PGA gel (△) and S72-netgel-6.5 (□), -17 (▽), and -36 (○) were immersed in pH = 2.0 aqueous solution. D_0 is the diameter of the hydrogels in 0.01 N NaCl aqueous solution (pH = 7.4), D is the diameter of the hydrogels after immersion in 0.01 N pH = 2.0 aqueous solution for the prescribed time, and D_∞ is the diameter of the equilibrium-swelling hydrogels in 0.01 N pH = 2.0 aqueous solution.

Table 2. Relationship between the Sulfonic Acid Molar Percentage and the Rate Constant k

sample	sulfonic acid molar percentage in hydrogel (%)	k (min^{-1})
γ-PGA hydrogel	0	8.89×10^{-2}
S72-netgel-6.5	6.5	1.10×10^{-1}
S72-netgel-17	17	3.42×10^{-1}
S72-netgel-36	36	4.44×10^{-1}

γ-PGA hydrogels and S72-netgels from pH = 7.4 to 2.0 analyzed by the D/D_0. All of the hydrogels indicated an equilibrium state after 120 min, and the diameters had shrunk by almost 60% from the pH = 7.4 condition. The swelling ratio of the hydrogels was dependent on the sulfonic acid molar percentage in the feed, and the S72-netgel including 36 mol % sulfonic acid (S72-netgel-36) showed the highest deswelling property (D/D_0 = 0.4). For a quantitative interpretation of the data, the deswelling process was fitted to an apparent first-order equation, as shown in Figure 2. The plots could be linearly fitted with a coefficient of variation of more than 0.98 for all cases, suggesting that the process was governed by a first-order rate step. The rate constants estimated from the slope of the linear plot are summarized in Table 2. The rate constant of the S72-netgel-36 was almost 5 times greater than that of the γ-PGA hydrogel, and the rate was dependent on the sulfonic acid molar percentage in the hydrogel.

Figure 3 shows the pH dependence swelling ratio of the γ-PGA hydrogel and S72-netgels. The pH-sensitive deswelling property of the γ-PGA hydrogel was observed at pH < 3.0, because the pK_a of γ-PGA is 2.27.[11] In the case of the S72-netgels, this deswelling property was observed even at a pH > 4.0, which is higher than the pK_a of γ-PGA. S72-netgels were composed of γ-PGA main network and interpenetrating polymer of γ-PGA-S72, and the deswelling properties of S72-netgels are due to the protonation of

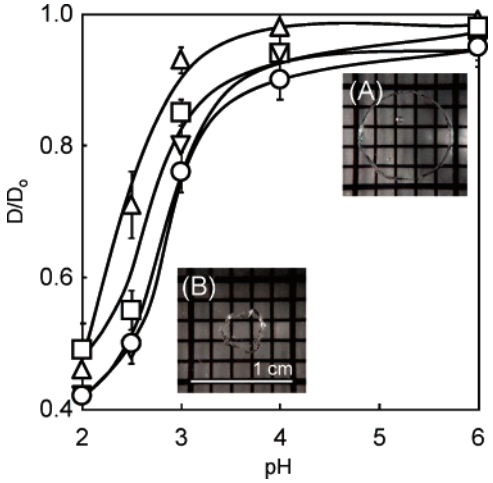

Figure 3. pH dependence swelling ratio of the γ-PGA gel (△) and S72-netgel-6.5 (□), -17 (▽), and -36 (○) in various pH solutions for 24 h (n = 3, means ± S. D.). D_0 is the diameter of the hydrogels in 0.01N NaCl aqueous solution (pH = 7.4), and D is the diameter of the hydrogels in various acidic solutions. The photographs are the S72-netgel-36 in pH = 6.0 (A) and 2.0 (B).

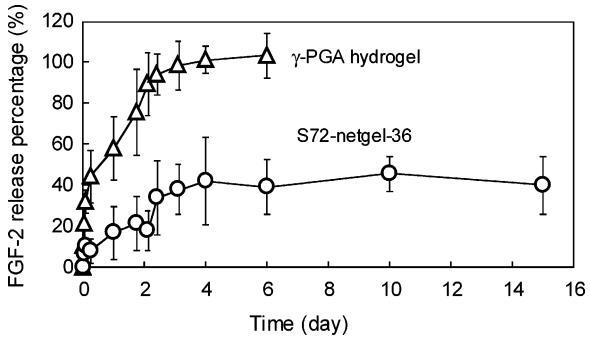

Figure 4. Release profiles of FGF-2 from the γ-PGA hydrogel (△) and the S72-netgel-36 (○) in Tris-HCl buffer (pH = 7.4) at 37 °C for 15 days. The amount of FGF-2 released was measured by the ELISA method. The FGF-2 release percentages are the average values from 3 experiments (n = 3, means ± SD).

carboxyl anions of γ-PGA main network. When the environmental pH is even higher than the pK_a, the carboxyl groups in the γ-PGA main network are presumably protonated by their neighboring sulfonic acids of γ-PGA-S72 interpenetrating polymer because the sulfonic acids increase the concentration of protons in water.[10] The swelling ratio of the hydrogels was dependent on the sulfonic acid molar percentage in the feed, and the S72-netgel-36 showed the deswelling property even at pH = 6.0 (D/D_0 = 0.94). These results indicated that the S72-netgel-36 has the highest and widest acidic pH-sensitive deswelling properties of all of these hydrogels.

These observations suggest that it is easy to control the swelling rate of pH-sensitive S72-netgels by altering the sulfonic acid molar percentage in the preparation of the hydrogels.

FGF-2 Releasing Properties of γ-PGA Hydrogel and S72-Netgel-36. We evaluated the FGF-2 release properties of the S72-netgel-36 and γ-PGA hydrogel. Figure 4 describes the FGF-2 release profiles from the γ-PGA hydrogel and S72-netgel-36 in Tris-HCl buffer (pH = 7.4) at 37 °C for 15 days. The amount of FGF-2 incorporated in the hydrogels was measured by the ELISA method, and 120 ± 26 and

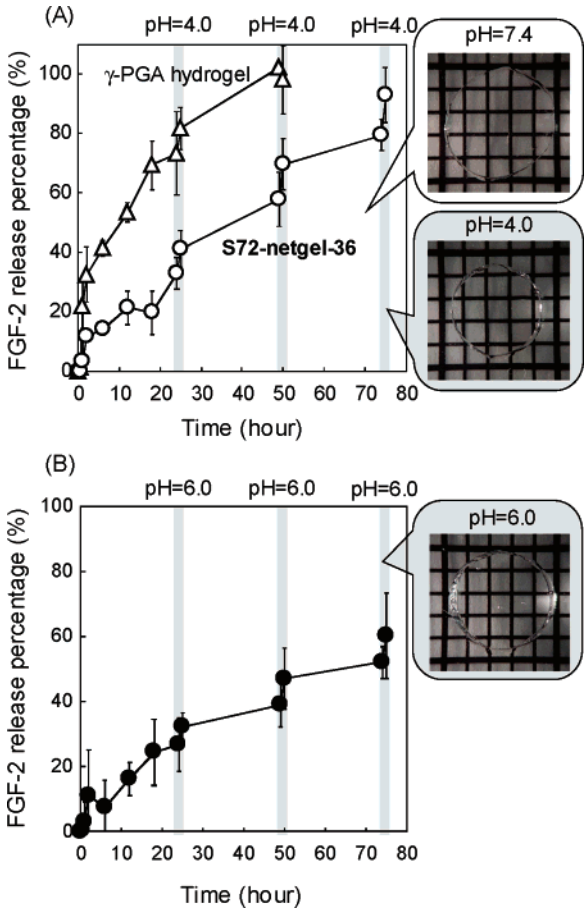

Figure 5. pH-sensitive stepwise release of FGF-2 from the γ-PGA hydrogel (△) and the S72-netgel-36 (○) at pH = 7.4 and 4.0 (A). The inserted photographs are the S72-netgel-36 at pH = 7.4 and 4.0. Stepwise release of FGF-2 from the S72-netgel-36 (●) at pH = 7.4 and 6.0 (B).

280 ± 38 ng of FGF-2 were incorporated, respectively, into the γ-PGA hydrogel and S72-netgel-36 (the volume of hydrogels is 7.85×10^{-2} cm^3) after 24 h of incubation in Tris-HCl buffer (pH = 7.4) containing 1.0 μg of FGF-2 at 4 °C. In the case of the γ-PGA hydrogel, almost 44% (48 ± 15 ng) of the FGF-2 was released within 6 h following the primary burst, and almost all of the FGF-2 was released within 2 days, because the γ-PGA hydrogel cannot strongly interact with FGF-2 as discussed in our previous reports.[9,10] However, the S72-netgel-36 still retained 60% (168 ± 40 ng) of the FGF-2 even after 15 days. We have reported that γ-PGA-S72 interacted strongly with FGF-2 by electrostatic interaction and hydrogen bonding like heparin or heparan-sulfate proteoglycan.[9] Due to this stable interaction with the γ-PGA-S72, FGF-2 would be retained in the S72-netgel-36 for 15 days, even though γ-PGA-S72 exists as an interpenetrating polymer in the S72-netgel-36. We previously reported that the S72-netgels retain their FGF-2 activity of γ-PGA-S72, which is an interpenetrating polymer in the S72-netgels.[10] The S72-netgel-36 containing the highest amount of γ-PGA-S72 in the feed had the highest FGF-2 and cell adhesion activities of all of the S72-netgels.

pH-Sensitive Controlled Release of FGF-2 from γ-PGA Hydrogel and S72-Netgel-36. Figure 5 shows the pH-sensitive stepwise release behavior of FGF-2 from the γ-PGA hydrogel and S72-netgel-36 at pH = 7.4 and 4.0 (A) and pH = 7.4 and 6.0 (B). In the case of the γ-PGA hydrogel, approximately 73% (88 ± 17 ng) of the FGF-2 was quickly released after 24 h of incubation, and 8.5% (10 ± 8.4 ng) of the FGF-2 was released after 1 h of incubation at pH = 4.0. Almost all of the FGF-2 was released from the γ-PGA hydrogel after 48 h, and γ-PGA hydrogel did not show the stepwise release of FGF-2 again. The release of FGF-2 from the γ-PGA hydrogel at pH = 4.0 seems to depend on the diffusion of FGF-2, because the γ-PGA hydrogel does not show any deswelling property at pH = 4.0 (Figure 2). On the other hand, 10.2% (29 ± 17 ng) of the FGF-2 was released from the S72-netgel-36 during the first incubation at pH=4.0, and the repetitive release of 12% (34 ± 24 ng) and 14% (39 ± 26 ng) of the FGF-2 was observed for each incubation at pH = 4.0 for 1 h. This was due to the pH-sensitive deswelling from pH = 7.4 to 4.0 as shown in the inserted photographs (Figure 5). The S72-netgel-36 can retain FGF-2 under pH-neutral conditions by its molecular interactions, and accordingly, FGF-2 was released from the S72-netgel-36 by physical deswelling under acidic conditions. In the case of other sulfonated S72-netgels, the stepwise release of FGF-2 was not observed. The reason for this phenomenon seems to be cause by the low incorporation ratio of γ-PGA-S72 in the hydrogels. In the case of S72-netgel-6.5 and -17, the molar ratio of γ-PGA-S72, which can interact with FGF-2, may be not enough to keep FGF-2 inside of the hydrogels. The S72-netgel-36 also showed pH-sensitive FGF-2 release at pH = 6.0, as shown in Figure 5(B). Totals of 5.6% (16 ± 11 ng), 7.9% (22 ± 16 ng), and 8.1% (23 ± 17 ng) of the FGF-2 were released from the S72-netgel-36 after 1 h of incubation at pH = 6.0, respectively. The acidic pH-sensitive controlled release of FGF-2 without denaturation was successfully achieved for the first time by a combination of FGF-2 activity and pH sensitivity.

Activity of FGF-2 Released from γ-PGA Hydrogel and S72-Netgel-36. To apply our pH-sensitive FGF-2 release system for neovascular treatment in an ischemic and inflamed area, it is desirable that the released FGF-2 still retains its activity. To confirm the activity of the released FGF-2, we evaluated the mitogenic activity of FGF-2 which was released from the hydrogels after 24 h (pH = 7.4) and 1 h (pH = 4.0) of incubation by L929 cell culture in serum-free Eagle's MEM medium (Figure 6). If the FGF-2 released from the hydrogels was deactivated by incubation in an acidic pH solution, the L929 cells will not be able to proliferate in serum-free medium containing the FGF-2. FGF-2 released from the S72-netgel-36 had adequate activity for cell proliferation, but the L929 cells could not proliferate well in serum-free medium containing FGF-2 released from the γ-PGA hydrogel. We have previously reported the high FGF-2 protective activity of γ-PGA-S72 in comparison to heparin under acidic and high heat conditions. About 40% of the FGF-2 was protected after 2 h of incubation at pH = 4.0 by their interaction with γ-PGA-S72.[9] Due to this protective effect of the γ-PGA-S72 in the S72-netgel-36, FGF-2 could retain its mitogenic activity even at a pH = 4.0 without denaturation. This result suggests that the S72-netgel-36 may be useful as a pH-sensitive controlled delivery

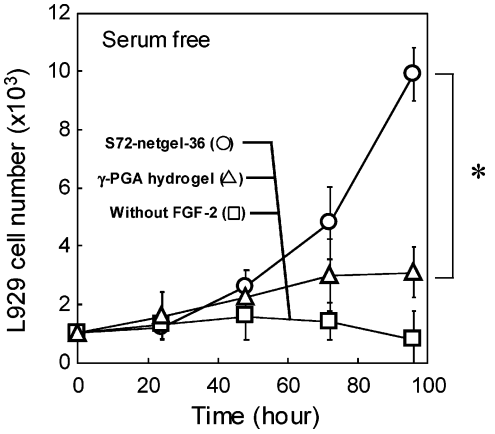

Figure 6. L929 cell proliferation was assayed in serum-free medium at 37 °C for 96 h with or without 10 ng of FGF-2, which was released from the γ-PGA hydrogel (△) and the S72-netgel-36 (○) after 24 h (pH = 7.4) and 1 h (pH = 4.0) of incubation. *Statistically significant difference ($p < 0.01$) using a two-sample *t* test for each comparison.

matrix for tissue engineering, such as the neovascular treatment of ischemia and inflammation.

Conclusions

In conclusion, we present here for the first time a novel strategy for the localized and pH-sensitive controlled delivery of FGF-2 without denaturation by a combination of FGF-2 activity and pH sensitivity. We believe that the design and preparation of biomaterials with growth factor activities and stimulus sensitivities is important for the stimulus-sensitive controlled release of growth factors in tissue engineering.

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References and Notes

(1) Hora, M. S.; Rana, R. K.; Nunberg, J. H.; Tice, T. R.; Gilley, R. M.; Hudson, M. E. *Pharm. Res.* **1990**, *7*, 1190−1194.
(2) Cohen, S.; Yoshioka, T.; Lucarelli, M.; Hwang, L. H.; Langer, R. *Pharm. Res.* **1991**, *8*, 713−720.
(3) Lee, K. Y.; Peters, M. C.; Anderson, K. W.; Mooney, D. J. *Nature* **2000**, *408*, 998−1000.
(4) Holland, T. A.; Tabata, Y.; Mikos, A. G. *J. Controlled Release* **2005**, *101*, 111−125.
(5) Hosaka, A.; Koyama, H.; Kushibiki, T.; Tabata, Y.; Nishiyama, N.; Miyata, T.; Shigematsu, H.; Takato, T.; Nagawa, H. *Circulation* **2004**, *110*, 3322−3328.
(6) Côté, M−F.; Laroche, G.; Gagnon, E.; Chevallier, P.; Doillon, C. J. *Biomaterials* **2004**, *25*, 3761−3772.
(7) Richardson, T. P.; Peters, M. C.; Ennett, A. B.; Mooney, D. J. *Nat. Biotechnol.* **2001**, *19*, 1029−1034.
(8) Matsusaki, M.; Serizawa, T.; Kishida, A.; Endo, T.; Akashi, M. *Bioconjugate Chem.* **2002**, *13*, 23−28.
(9) Matsusaki, M.; Serizawa, T.; Kishida, A.; Akashi, M. *Biomacromolecules* **2005**, *6*, 400−407.
(10) Matsusaki, M.; Serizawa, T.; Kishida, A.; Akashi, M. *J. Biomed. Mater. Res. Part A* **2005**, *73A*, 485−491.
(11) Kubota, H.; Matsunobu, T.; Uotani, K.; Takebe, H.; Satoh, A.; Tanaka, T.; Taniguchi, M. *Biosci. Biotechnol. Biochem.* **1993**, *57*, 1212−1217.
(12) Tachibana, Y.; Kurisawa, M.; Uyama H.; Kobayashi S. *Biomacromolecules* **2003**, *4*, 1132−1134.
(13) Matsusaki, M.; Hiwatari, K.; Higashi, M.; Kaneko, T.; Akashi, M. *Chem. Lett.* **2004**, *33*, 398−399.
(14) Marui, A.; Kanematsu, A.; Yamahara, K.; Doi, K.; Kushibiki, T.; Yamamoto, M.; Itoh, H.; Ikeda, T.; Tabata, Y.; Komeda, M. *J. Vasc. Surg.* **2005**, 41, 82−90.
(15) Yajima, S.; Ishikawa, M.; Kubota, T.; Moroi M.; Sugi, K.; Namiki, A. *Int. Heart J.* **2005**, *46*, 289−301.
(16) Lahama, R. J.; Sellke, F. W.; Edelman, E. R.; Pearlman, J. D.; Ware, J. A.; Brown, D. L.; Gold, J. P.; Simons, M. *Circulation* **1999**, *100*, 1865−1871.
(17) Nakajima, H.; Sakakibara, Y.; Tambara, K.; Iwakura, A.; Doi, K.; Marui, A.; Ueyama, K.; Ikeda, T.; Tabata, Y.; Komeda, M. *J. Artif. Organs* **2004**, 7, 58−61.
(18) Tabata, Y.; Hijikata, S.; Ikada, Y. *J. Controlled Release* **1994**, *31*, 189−199.
(19) Na, K.; Lee, K. H.; Bae, Y. H. *J. Controlled Release* **2004**, *97*, 513−525.
(20) Tannock, I. F.; Rotin, D. *Cancer Res.* **1989**, *49*, 4373−4384.
(21) Zhang, J.; Peppas, N. A. *Macromolecules* **2000**, *33*, 102−107.
(22) Huh, K. M.; Kumashiro, Y.; Ooya, T.; Yui, N. *Polym. J.* **2001**, *33*, 108−111.
(23) Kumashiro, Y.; Lee, W. K.; Ooya, T.; Yui, N. *Macromol. Rapid Commun.* **2002**, *23*, 407−410.

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