International Journal of Pharmaceutics 414 (2011) 193–202

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

The characterization of protein release from sericin film in the presence of an enzyme: Towards fibroblast growth factor-2 delivery

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ARTICLE INFO

Article history:
Received 10 March 2011
Received in revised form 23 April 2011
Accepted 11 May 2011
Available online 17 May 2011

Keywords:
Sericin
Sustained release
Film
Biodegradation
FGF-2
Bone defection

ABSTRACT

Aqueous preparations of silk protein (sericin) films were prepared to evaluate their biodegradation properties. In the absence of trypsin, sericin film swelled rapidly, kept its shape, and remained unaltered for 28 days or longer due to form β-sheet structures. In the presence of trypsin, sericin film gradually degraded; since the rate depended on the concentration of trypsin, the films likely underwent enzymatic hydrolysis. Sericin film incorporating the model protein drug fluorescein isothiocyanate–albumin (FA) also gradually degraded in the presence of trypsin and resulted in the sustained release of FA for 2 weeks or longer; in contrast, FA release was quite slow in the absence of trypsin. It is expected that sericin film has potential as a biodegradable and drug-releasing carrier. To evaluate the practical applicability of sericin film for the repair of defective tissues, fibroblast growth factor-2 (FGF-2) was incorporated into sericin films and the films were implanted on skull defects in rats. Whereas FGF-2 release was suppressed in the absence of trypsin *in vitro*, it appears that FGF-2, immobilized by ionic interactions between sericin and FGF-2, can be sustained-released *in vivo* from films incorporating 2500 or 250 ng of FGF-2 to support the growth of tissue around wounds.

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1. Introduction

Silk is a natural protein derived from the silkworm *Bombyx mori*, and is primarily composed of sericin (SC) and fibroin. SC constitutes 25–30% of silk protein and covers the fibroin fibers like glue to form the cocoon (Zhang, 2002). The SC protein is composed of 18 kinds of amino acids, most of which are polar (e.g., hydroxyl, acidic, or basic amino acids; Komatsu and Yamada, 1975). Cocoon SC from *B. mori* mainly consists of three polypeptides having molecular masses of 400, 250, and 150 kDa as estimated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), which correspond to the SC present in the middle, anterior, and posterior part of the middle silk gland (Takasu et al., 2002, strain of silkworm, C145 × J140; Teramoto et al., 2005, strain of silkworm, Sericin Hope). Amino acid analysis of SC (Takasu et al., 2002) showed that all three SC polypeptides have high contents of serine (Ser, 33.2–39.0%), glycine (Gly, 14.1–16.0%), and aspartic acid/asparagine (Asp/Asn, 11.3–15.7%). The structural analysis and cloning of SC genes *Ser1* and *Ser2* (*Src-2*) have been described (Okamoto et al., 1982; Gamo, 1982; Michaille et al., 1990; Garel et al., 1997). The most abundant component is the largest SC (400 kDa), which corresponds to the Ser1C protein encoded by the *Ser1* gene (Takasu et al., 2002). A repetitive 38-amino acid sequence rich in Ser dominates a large part of the Ser1C protein and is predicted to have a strong tendency to form a β-sheet structure. Another part of the Ser1C protein is hydrophilic, and has a high content of charged residues including acidic (glutamic acid (Glu) and Asp) and basic (lysine (Lys) and arginine (Arg)) amino acids (Garel et al., 1997). In contrast, the 250 kDa SC polypeptide, which corresponds to the S-2 protein encoded by the *Src-2* gene, has less β-sheet-forming propensity and higher hydrophilicity. Thus, such an acid composition gives SC a unique protein structure.

Several biocompatible and biodegradable drug-release materials have previously been developed. Some, including synthetic polymers such as poly(lactic-co-glycolic acid), natural polymers such as gelatin and chitosan, and combinations of these (Okada et al., 1994; Tabata and Ikada, 1999; Kushibiki et al., 2006; Kanazawa et al., 2009; Bhattarai et al., 2010; Takekawa et al., 2010), are currently in medical use. Although such materials have excellent characteristics as biomaterials, their applications have been limited due to low compatibility with certain drugs, the use of large amounts of organic solvents or toxic cross-linkers, and problems arising from bovine spongiform encephalopathy. Therefore, the development of better options for drug-releasing materials with sufficient biocompatibility is important. Recently, it was reported that SC displays no immunogenicity in *in vivo* experiments, and that it can thus be used effectively for biomedical applications (Zhang

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doi:10.1016/j.ijpharm.2011.05.033

et al., 2006a). Furthermore, SC films showed low inflammatory response, as shown by assaying TNFα release (Dash et al., 2009). SC is expected to be a biocompatible material.

In previous studies of the functions and applications of SC for medical or cosmetic use, SC hydrolysate, which is a waste product of silk scouring processes such as alkali treatment and high-pressure steam heating in the silk spinning and weaving industries, has been reported to have various functions, including as a cell attachment enhancer (Minoura et al., 1995; Tsubouchi et al., 2005), moisturizer (Zhang, 2002), UV-resistance agent (Dash et al., 2008a), and antioxidant agent (Kato et al., 1998; Dash et al., 2008b). Recently, a serum-free medium using SC hydrolysate was developed for cell culture and was demonstrated to be superior to commercial serum-free media (Kobayashi et al., 2009). In addition, Miyamoto et al. (2009) reported that SC is useful for cryopreservation of human adipose tissue-derived stem/progenitor cells. Several dosage forms compatible with SC hydrolysate being used as a biomaterial have been reported, such as a cross-linked film and sponge-like scaffold of SC/gelatin and SC/polyvinyl alcohol (PVA) (Mandal et al., 2009; Aramwit et al., 2010), and an SC cream reported to have practical use as a wound dressing (Aramwit and Sangcakul, 2007). Intact or partially degraded high molecular weight SC is extracted by dissolving *B. mori* cocoons (Sericin Hope and N124 × C124) in lithium bromide (LiBr) aqueous solution or heating it for tens of minutes in water (Teramoto et al., 2005; Mase et al., 2006; Komatsu, 1975). This SC provides stable matrix-like hydrogels, porous materials, and film with properties for forming β-sheet structures (Kundu et al., 2008). A film has been prepared from SC aqueous solution using the casting method (Teramoto and Miyazawa, 2005; Teramoto et al., 2008), a moldable gel has been prepared by aging SC solution using a specific process (Zhu et al., 1995; Teramoto et al., 2005), a hydrogel sheet (like the cream mentioned above) was reported to have practical use as a wound dressing (Tsubouchi, 1999; Teramoto et al., 2008), and a porous sponge has been prepared by freeze-drying (Tao et al., 2005). Thus, SC has various functions useful in medicine, and intact or partially degraded SC can take on various forms when prepared by aqueous processes without the use of a cross-linker.

In the biodegradation mechanisms of biomaterials, polymer biodegradation occurs by physical mechanisms such as sorption, swelling, dissolution, mineralization, crystallization, fatigue fracture, impact fracture, and wear, and chemical mechanisms such as hydrolysis, enzymolysis, oxidation, and oxidative chain cleavage (Kopecek and Ulbrich, 1983). No single mechanism should be considered as causing biodegradation. During degumming of silk fabric, SC is hydrolyzed by proteases and removed from silk fibroin (Freddi et al., 2003), so enzymatic degradation may be a major degradation mechanism of SC protein. However, the enzymatic biodegradation of SC fabricated into a dosage form, and its drug release properties, have been poorly studied. In this study, SC films as a biodegradable carrier for drug-release were prepared using high molecular weight SC, and to evaluate the biodegradation of the film, its enzymatic degradation and the release properties of a model protein incorporated into SC film were evaluated using trypsin, a hydrolyzing protease, *in vitro*.

Fibroblast growth factor-2 (FGF-2) has a strong proliferative effect on endothelial cells, osteocytes, and chondrocytes (Connolly et al., 1987; Gospodarowicz et al., 1987). FGF-2 exerts its proangiogenic activity by interacting with various endothelial cell surface receptors. Also, cross-talk among FGFs, VEGFs, and inflammatory cytokines may play a role in the modulation of angiogenesis in different pathological conditions, including bone healing (Presta et al., 2005). The administration of FGF-2 has shown therapeutic potential for tissue regeneration. However, the bioactivity of FGF-2 is unreliable when FGF-2 is administrated in solution because of its short retention time at wound sites and short half-life caused by its susceptibility to enzymatic degradation *in vivo*. To enhance the therapeutic efficacy of FGF-2, a drug delivery system allowing sustained and localized release of FGF-2 is needed (Tabata, 2005). Ionic interactions between a charged protein drug and a charged drug-release material can form the basis of an effective drug delivery system (Biondi et al., 2008). For example, polyion complexation between an acidic gelatin hydrogel and FGF-2 (Tabata and Ikada, 1999) provides a drug delivery system in which the hydrogel slowly degrades and FGF-2 is released for an extended period of time, leading to prolonged vascularization (Tabata et al., 1999). In another study, a polylactic and polyglycolic acid copolymer, amalgamated with a gelatin sponge, released incorporated FGF-2 and bone morphogenic protein (BMP). As a result, skull bone damage in rats was diminished by enhanced calcification due to the osteoinductive activity of BMP, which was enhanced by FGF-2 (Tanaka et al., 2006). We previously reported that fluorescein isothiocyanate–albumin (FA), a charged protein model drug, was released for a longer period of time from SC film, gel, and sponge than fluorescein isothiocyanate–dextran, an uncharged model drug (Nishida et al., 2010). Taken together, SC is expected to be promising as a biodegradable and drug-releasing material, and has useful functions by forming matrices and binding charged protein drugs such as FGF-2 through ionic interactions with its polar side chains.

The present study aimed to evaluate the biodegradation of SC film and the release properties of a protein drug in enzymatic conditions *in vitro*. In addition, to evaluate the practicality of SC film for regenerative medical therapy, FGF-2 was incorporated in SC films, its sustained release was quantified, and the ability of these films to promote the healing of rat skull bone defects was assessed.

2. Materials and methods

2.1. Materials

Intact SC was obtained from fibroin-deficient cocoons spun by the transgenic *B. mori* silkworm strain Sericin Hope (Sericin Hope SHC; purity of SC, 98.5%; Kougensha Co., Ltd., Nagano, Japan) (Mase et al., 2006). Fluorescein isothiocyanate–albumin (FA, Sigma–Aldrich Japan Co., Ltd., Tokyo, Japan) and human recombinant FGF-2 (Millipore, Temecula, CA) were used as model drugs. Glycerin (GL, Wako Pure Chemicals Industries Ltd., Osaka, Japan) was used as a plasticizer to allow easy cutting of the SC film.

2.2. Preparation of SC solution

To prepare SC solution (2%), Sericin Hope powder (2:100, w/v) was dispersed and dissolved in purified water at 100 °C for 20 min, centrifuged at 3000 rpm for 5 min to remove insoluble SC and other insoluble components, then cooled to room temperature. To confirm the actual concentration of SC extracted in the solution, a sample of the SC solution was dried at 40 °C for 1 day. The dried mass was weighed and the concentration of SC in 2% SC solution was calculated using the following equation:

$$\%SC = \frac{W_a}{W_b} \times 100$$

where W_a is the weight of the dried mass from the SC solution, and W_b is the weight of SC powder added in water. The 2% SC solution was diluted 1:1 with purified water (1% SC solution). To prepare a practical SC film, the plasticizer GL was mixed with 2% or 1% SC solutions at a ratio of 0.2:100, GL: SC solution, w/v.

2.3. Preparation of SC film for swelling evaluation

To evaluate the swelling properties of SC films, films with and without GL were prepared. To prepare SC film, 2 ml of 2% and 1% SC solution containing or not containing GL was cast on polyethylene

Table 1
Formulations of SC films for *in vitro* and *in vivo* studies.

Formulations	Materials				Film size	
	FA (mg/unit)	FGF-2 (ng/unit)	SC solution[a]		*In vitro* study	*In vivo* study
			SC (%, w/v)	GL (%, w/v)		
2% SC film	–	–	2	–	10 mm × 10 mm[b] and 23 mmΦ[c]	–
1% SC film	–	–	1	–		
2% SC-GL film	–	–	2	0.2	10 mm × 10 mm[b]	
1% SC-GL film	–	–	1	0.2		
SC-FA film	1	–	2	–	23 mmΦ[d]	–
SC-GL-FA film	1	–	2	0.2		
2% SC-FGF2500 film	–	2500	2	0.2	10 mmΦ[d]	5 mm × 5 mm[e]
2% SC-FGF250 film	–	250	2	0.2		
2% SC-FGF25 film	–	25	2	0.2		
1% SC-FGF2500 film	–	2500	1	0.2		
1% SC-FGF250 film	–	250	1	0.2		
1% SC-FGF25 film	–	25	1	0.2		
2% SC-FGF0 film	–	–	2	0.2		

SC, sericin; FA, fluorescein isothiocyanate–albumin; FGF-2, fibroblast growth factor-2; GL, glycerin.
[a] The water in the SC films was removed by drying.
[b] For swelling evaluation, 20 mm × 20 mm films were trimmed into 10 mm × 10 mm.
[c] For enzymatic degradation testing.
[d] For release testing.
[e] For histological evaluation, 20 mm × 20 mm films were trimmed into 5 mm × 5 mm.

dishes (20 mm × 20 mm) and dried at 40 °C for 1 day (Table 1). The films were cut into 10 mm × 10 mm squares.

2.4. Swelling properties of SC films

The SC films were accurately weighed in the dry state and then immersed in 1 ml of phosphate-buffered saline (PBS, pH 7.4). At various time intervals, the buffer was carefully removed and the amount of buffer in the films was determined precisely by weighing the film in the swollen state. The percentage swelling of the films at equilibrium was calculated using the following equation:

$$\%\text{swelling of SC film} = (W_c - W_d)/W_d \times 100$$

where W_c is the weight of the swollen test sample and W_d is the weight of the dried sample before testing.

2.5. Preparation of SC film for enzymatic degradation testing

To evaluate the enzymatic degradation of SC films, films were prepared by dispensing 2 ml of 2% or 1% SC solution into test tubes (23-mm inner diameter). 2% and 1% SC film were obtained by drying each solution in the tubes at 40 °C for 1 day (Table 1).

2.6. Enzymatic degradation of SC films

The enzymatic degradation of SC films was evaluated using bovine pancreatic trypsin (EC. 3.4.21.4, Sigma–Aldrich Japan Co., Ltd., Tokyo, Japan). The films were incubated at 37 °C in 1 ml of 0.05 M Tris–hydrochloric buffer (TB, pH 7.8) containing 0.01 mg of trypsin for 1 day and 1.0 mg of trypsin for 28 days. The trypsin solution was replaced daily with freshly prepared solution. Films were also incubated in TB without trypsin as a control. Following incubation, the films were washed twice with purified water, then air-dried at 37 °C for 1 day. The weights of the films were measured and the films were observed by scanning electron microscopy (SEM, JSM-5200LV, JEOL, Tokyo, Japan). The percentage of remaining film in each sampling point was calculated using the following equation:

$$\%\text{remaining of SC film} = \frac{W_e}{W_f} \times 100$$

where W_e is the weight of the dried test sample after testing and W_f is the weight of the dried test sample before testing.

To analyze the chemical properties of SC treated in enzymatic conditions, the molecular weight of SC in films incubated in TB with or without trypsin (0.01 mg/ml and 1.0 mg/ml) for 1 and 3 days was determined by SDS-PAGE. The test samples were washed twice with purified water after the incubation, dissolved in 8 M LiBr solution, and dialyzed against distilled water for 2 days (the water was changed three times per day) by the method of Teramoto et al. (2005). The dialyzed SC solutions were electrophoresed on 5–20% polyacrylamide gels (C10L, ATTO Corporation, Tokyo, Japan) and the proteins were stained with Coomassie Brilliant Blue R-250.

2.7. Preparation of SC film for release testing of FA

To evaluate FA release from SC films, FA (0.5 mg/ml) was dissolved in either 2% SC solution containing or not containing GL (50 ml), and then 2 ml of SC solution containing 1.0 mg FA was dispensed into test tubes (23-mm inner diameter). Films were obtained by drying each solution in the tubes at 40 °C for 1 day (SC-FA film and SC-GL-FA film in Table 1).

2.8. Release properties of FA from SC films

To evaluate the release rate of FA from the SC films, 1 ml of TB (pH 7.8) with and without trypsin (0.01 and 1.0 mg/ml) was added to each SC preparation in a test tube and incubated at 37 °C. The medium was replaced daily. The released FA in test media for each sampling time was measured by ultraviolet–visible absorption spectrophotometry at 494 nm (Spectra Max, Molecular Devices, Sunnyvale, CA, USA), and the released FA amount in each fraction was used to calculate release profiles.

2.9. Preparation of SC film for release testing of FGF-2

To evaluate FGF-2 release from SC films, 100 μl of 2% or 1% SC solution containing GL was dispensed into the wells of a 48-well plate (10-mm inner diameter), and then 25 μl of 0.1 M phosphate buffer (pH 6.8) containing 2500, 250, or 25 ng of FGF-2 was added and mixed with the SC solution in the wells and dried at 25 °C, 30% relative humidity, for 1 day (2% and 1% SC-FGF2500, 250, 25 film in Table 1).

2.10. Release properties of FGF-2 from SC films

The rate of FGF-2 release from the SC films was measured using an enzyme-linked immunosorbent assay (ELISA). Cell culture medium consisting of alpha minimum essential medium (αMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 1% albumin and 1% penicillin/streptomycin based on the method of Macdonald et al. (2010) was used as the test medium. One hundred microliters of the cell culture medium was added to each well with a cast SC film containing FGF-2 (2500, 250, or 25 ng/unit) and incubated at 37 °C. The medium was replaced daily with 100 μl of flesh medium. Samples were analyzed using ELISA development kits (Quantikine, Human FGF basic immunoassay, R&D Systems, Inc., Minneapolis, MN, USA), and then the release rate of FGF-2 was measured by subtracting readings at 540 nm from the readings at 494 nm with ultraviolet–visible absorption spectrophotometry (Spectra Max, Molecular Devices, Sunnyvale, CA, USA) according to manufacturer's instructions.

2.11. Animals for in vivo study

Seven-week-old male Sprague-Dawley rats were purchased from Japan SLC, Inc. (Shizuoka, Japan). The rats were housed under standard conditions of temperature (22–24 °C), humidity (40–60%), and 12-h-light/dark-cycles with the light period starting at 08:00. Food and water were supplied *ad libitum*. All experiments with animals were carried out in accordance with a protocol approved by the Animal Care and Ethics Committee of Tokyo University of Pharmacy and Life Sciences (TUPLS).

2.12. Preparation of SC film for the in vivo study

SC films containing FGF-2 were prepared for *in vivo* study. FGF-2 (50:500, w/v) was dissolved in 0.1 M phosphate buffer (pH 6.8). The solution (400, 40, 4, or 0 μl) containing FGF-2 (respectively 40, 4, 0.4, or 0 μg) was mixed with 1.6 ml of 2% SC solution containing GL; in addition, 40 μl of FGF solution was mixed with 1.6 ml of 1% SC solution containing GL. Each mixture was cast on polyethylene dishes (2 cm × 2 cm) and dried at 25 °C with 30% relative humidity for 1 day. Films containing 2500, 250, 25, or 0 ng of FGF-2 (2% SC-FGF0, 2500, 250, 25, 0 film and 1% SC-FGF250 film in Table 1) were cut into 5 mm × 5 mm squares prior to implantation onto the skull bones of rats. The films were prepared with sterile purified water and aseptic containers at a clean bench.

2.13. Implantation of SC films containing FGF-2 on skull bone defects in rats

Rats were anesthetized by intraperitoneal administration of sodium pentobarbital (50 mg/kg), and an anteroposteriorly directed incision about 10 mm long was made in the scalp. The periosteum of the skullcap was ablated, and a full thickness standardized trephine defect with a diameter of 3 mm was made bilaterally in the parietal bone using a low-speed trephine burr. One side of this artificial defect was covered either with SC film containing FGF-2 or not containing FGF-2 ($n = 7$ in 2% SC-FGF250, 0 and 1% SC-FGF250 film groups; $n = 4$ in SC-FGF2500 and 25 film groups). The other side was left untreated as a control. After implantation, the ablated periosteum was replaced and the skin was sutured.

The rats were sacrificed 2 weeks after implantation. The grafted bone defects were dissected along with the surrounding bone tissue and examined visually.

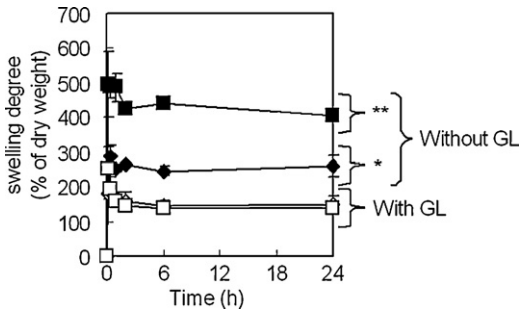

Fig. 1. The degree of swelling (% of dry weight) of SC film with or without glycerin (GL) as a plasticizer in phosphate-buffered saline (PBS, pH 7.4). (♦) 2% SC film; (◊) 2% SC-GL film; (■) 1% SC film; (□) 1% SC-GL film. Each bar represents the mean ± SD ($n = 3$). Statistical comparison was made by two-sided *t*-test ($\alpha = 0.05$) at each sampling point. *$P < 0.01$: significant difference versus 2% SC-GL film and 1% SC film (from 10 min to 24 h). **$P < 0.01$: significant difference versus 1% SC-GL film and 2% SC film (from 10 min to 24 h).

2.14. Histological evaluation

The removed skull bones treated with SC films were fixed in 4% phosphate-buffered formalin, demineralized with OSTEOSOFT® (Merck Chemicals Japan, Tokyo, Japan) for 3 days, and then embedded in paraffin. Horizontal or vertical sections were routinely stained with hematoxylin and eosin. These sections were observed with a microscope (BZ-8100, Keyence Japan, Osaka, Japan).

3. Results and discussion

3.1. SC solution

Sericin Hope powder (2:100, w/v) was dispersed and dissolved in purified water at 100 °C for 20 min. The dried SC mass obtained from the solution was 1.28% (w/v). This indicated that 64% (w/w) of the SC powder was dissolved in water by heating, and the other insoluble portion was removed by centrifugation.

3.2. Swelling properties of SC films

Films made of SC alone exhibit elastic distortion and are easily broken during the drying process, showing that their use is not practical. It has been reported that biocompatible plasticizers, such as glycerin, confer significant flexibility and elasticity to the protein films (Sobral et al., 2002; Hernandez et al., 2008). In our previous study, we reported that dried SC film containing a moisture-retaining plasticizer such as GL or D-sorbitol showed that the protein adopted a β-sheet structure and withstood high mechanical stress, while dried protein in SC-alone film had a random coil structure and the plasticized SC film exhibited improved film-forming properties and tensibility (Nishida et al., 2010). In the current study, 2% and 1% SC films containing or not containing GL immersed in PBS swelled rapidly, retained their shape, increased in volume by 150–300% in the 2% SC films, and 140–400% in the 1% SC films (Fig. 1), and had elastic properties. The amounts of swelling by SC-GL films were significantly smaller than those of SC films not containing GL after 10 min. Since GL is highly soluble in the test medium, it is likely that SC film released GL rapidly, thereby reducing its ability to retain water. The amounts of swelling by 1% SC films were significantly larger than those of 2% SC films, though there was not a significant difference between 1% and 2% SC films containing GL. The cause of this is not known; however, 1% SC films retained water more easily than 2% films because 1% SC films more easily elongated in PBS.

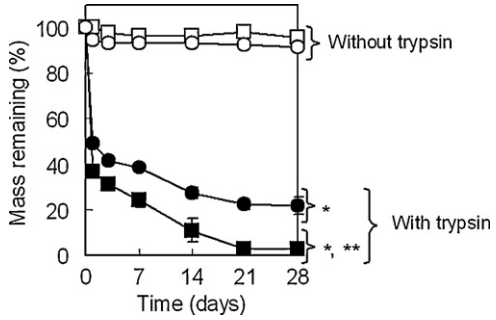

Fig. 2. *In vitro* degradation of SC film incubated in Tris–hydrochloric buffer (TB, pH 7.8) with and without trypsin at 37 °C. (○) 2% SC film incubated in TB without trypsin; (□) 1% SC film incubated in TB without trypsin; (●) 2% SC film incubated in TB with 1.0 mg/ml of trypsin; (■) 1% SC film incubated in TB with 1.0 mg/ml of trypsin. Each bar represents the mean ± SD ($n = 3$). Statistical comparison was made by two-sided t-test ($\alpha = 0.05$) at each sampling point. $^{*}P < 0.01$: significant difference versus TB without trypsin (from 1 to 28 days). $^{**}P < 0.01$: significant difference versus 2% SC film (from 1 to 28 days).

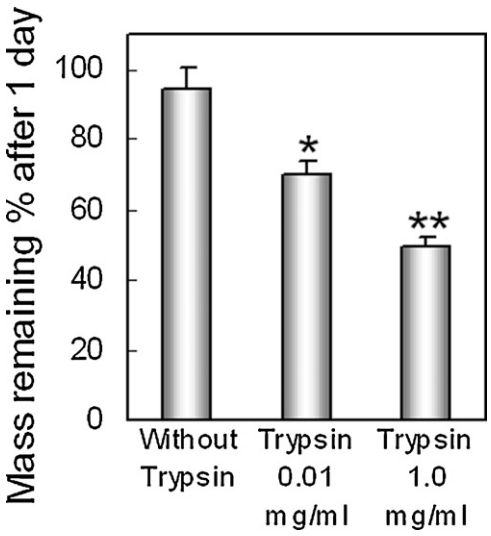

Fig. 3. *In vitro* degradation of 2% SC film in Tris–hydrochloric buffer (TB, pH 7.8) with 0, 0.01, or 0.1 mg/ml of trypsin at 37 °C. Each bar represents the mean ± SD ($n = 3$). Statistical comparison was made by two-sided t-test ($\alpha = 0.05$). $^{*}P < 0.01$: significant difference versus TB without trypsin. $^{**}P < 0.01$: significant difference versus TB with 0.01 mg/trypsin.

3.3. Enzymatic degradation of SC films

Aramwit et al. (2010) reported that a non-crosslinked (low molecular weight) SC hydrolysate/PVA scaffold completely dissolved in PBS in less than 30 min, while a high concentration of the cross-linker, genipin, inhibited the dissolution of SC in PBS. Crosslinked SC hydrolysate/gelatin scaffold degraded gradually over a period of 28 days or more (Mandal et al., 2009). In the present study, we evaluated the *in vitro* degradation of SC films (2% and 1% SC film) submerged in buffer with or without 1.0 mg/ml of trypsin; the enzyme solution was changed daily. Trypsin is a hydrolyzing protease found in the digestive system of many vertebrates (Rawlings and Barrett, 1994). The weight of SC films incubated without trypsin decreased only marginally over 28 days, whereas the weights of 2% and 1% SC films incubated for 1 day in the presence of 1.0 mg/ml trypsin decreased to 49% and 37% of their original weights, respectively, although further degradation after day 3 was gradual (Fig. 2). The weight loss rate in 1% SC film was significantly faster compared with 2% SC film throughout the testing time. Thus, the weight loss of SC films was dependent on their SC concentration. After that, the weight loss of SC films was TP 1.0 mg/ml >TP 0.01 mg/ml >without TP (Fig. 3). The weight loss of SC films was also dependent on the trypsin concentration in the medium. SDS-PAGE of SC film incubated in the absence of trypsin showed a high-molecular weight band corresponding to SC (>250 kDa, A and D in Fig. 4) in the intact film (not shown). SC in film incubated with 1.0 mg/ml trypsin for 1–3 days was degraded to fragments with molecular weights between 10 and 70 kDa (C and F in Fig. 4). SC film incubated in 0.01 mg/ml trypsin medium for 3 days had a distribution of molecular weights (E in Fig. 4) slightly lower than that of SC incubated in the absence of trypsin (B in Fig. 4).

Cocoon SC from *B. mori* mainly consists of three polypeptides having molecular masses of 400, 250, and 150 kDa as estimated by SDS-PAGE, which corresponds to the SC present in the middle, anterior, and posterior part of the middle silk gland (Takasu et al., 2002, strain of silkworm, C145 × J140; Teramoto et al., 2005, strain of silkworm, Sericin Hope). The most abundant component is the largest SC (400 kDa), which corresponds to the Ser1C protein (331 kDa) encoded by the *Ser1* gene (Takasu et al., 2002). A major part of the Ser1C protein is comprised of peptides 3, 6, and 8 encoded by exons 3, 6, and 8 of the *Ser1* gene (Garel et al., 1997). Peptides 6 and 8 contain about 70 repeats of a characteristic 38-amino acid repetitive motif (SSTGSSSNTDSNSNSVGSSTSGGSSTYGYSSNSRDGSV) rich in Ser (Michaille et al., 1990), whereas peptide 3 serves as a hydrophilic region with a high content of charged residues (Garel et al., 1997). Huang et al. (2003) cloned, expressed, and purified a

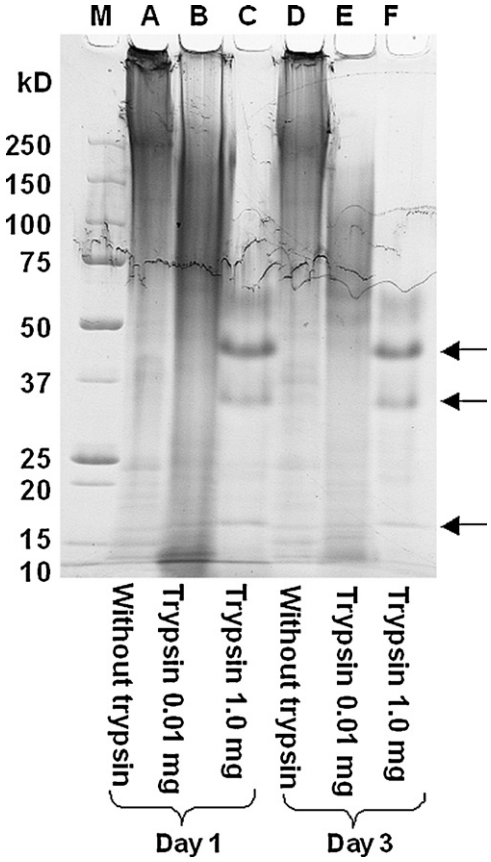

Fig. 4. SDS-PAGE results for SC incubated in various degradation conditions at 37 °C: (A) incubated in Tris–hydrochloric buffer (TB, pH 7.8) without trypsin, for 1 day; (B) TB with 0.01 mg/ml of trypsin, for 1 day; (C) TB with 1.0 mg/ml of trypsin, for 1 day; (D) TB without trypsin, for 3 days; (E) TB with 0.01 mg/ml of trypsin, for 3 days; (F) TB with 1.0 mg/ml of trypsin, for 3 days; (M) molecular weight markers; arrows, degraded SC protein bands (MW: 15–50 kDa).

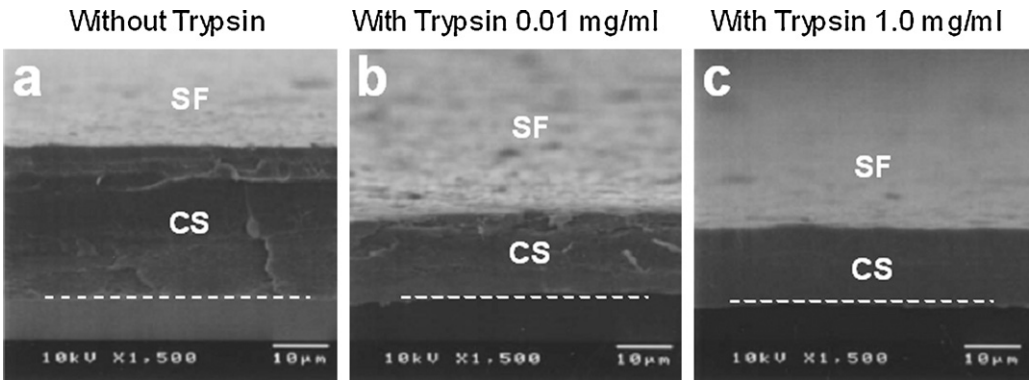

Fig. 5. SEM photographs of 2% SC film incubated for 3 days in Tris–hydrochloric buffer (TB, pH 7.8) with 0 (a), 0.01 (b) or 0.1 mg/ml (c) of trypsin at 37 °C. CS, cross-section of SC film; SF, surface of SC film; dotted line, basement of SC film adhered to the sample tube; scale bar, 10 μm.

recombinant SC-like protein of different molecular weights (17.4, 31.9, and 46.5 kDa) based on the 70 repeats of the 38-amino acid repetitive motif of native SC, and demonstrated the proteins' conformational transitions to β-sheets. These molecules have been speculated to correspond to peptides 6 and 8. The hydrophilic region (peptide 3) contains 13% of Glu + Asp and 15% of Lys + Arg (Garel et al., 1997). Trypsin cleaves the peptide chains mainly at the carboxyl side of Lys and Arg. In our results, interestingly, SC in the presence of a high trypsin concentration (1.0 mg/ml) provided bands that correspond to the bands of the recombinant protein as 17.4, 31.9, and 46.5 kDa after 1 day (arrows in C and F, Fig. 4). We speculated that the SC molecules were peptides 6 and 8 that form β-sheet structures and remain intact, while other SC peptide bonds in hydrophilic regions are selectively cleaved. Freddi et al. (2003) studied the degumming process of silk fabric and utilized enzymatic degradation of SC, and reported that hydrolytic degradation of SC took place by selective peptide bond cleavage, and enzyme-treated SC peptides ranging from 5 to 20 kDa were obtained with alkaline and neutral proteases (3374-L, Oxidative-stable endopeptidase; GC-897-H, Bacterial high alkaline strain; 3273-C, Papain, thiol protease). This resulted in main chain fission and formation of a range of small sized peptides. Teramoto et al. (2007) suggested, based on ^{13}C NMR spectroscopy data, that degradation of intact SC is caused by heat hydrolysis of its hydrophilic region as peptide 3, and that the SC Asp residues might be hydrolyzed preferentially during heat treatment in the solution state. Hydrolytic cleavage of SC might easily occur in the hydrophilic regions. At day 3, digestion with 0.01 mg/ml trypsin (E, Fig. 4) showed a smear of smaller SC molecules compared to day 1 (B, Fig. 4). In the lower concentration of trypsin (0.01 mg/ml), the SC degradation rate to small molecular sizes may be slower than that in high concentration of trypsin (1.0 mg/ml).

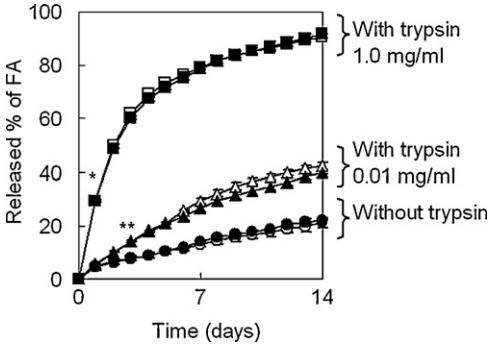

Fig. 6. *In vitro* release profiles of fluorescein isothiocyanate–albumin (FA) from SC film containing or not containing glycerin (GL) in various degradation conditions at 37 °C. 2% SC film: (○) incubated in Tris–hydrochloric buffer (TB, pH 7.8) without trypsin; (△) TB with 0.01 mg/ml of trypsin; (□) TB with 1.0 mg/ml of trypsin. 2% SC-GL film: (●) incubated in TB, pH 7.8 without trypsin; (▲) TB with 0.01 mg/ml of trypsin; (■) TB with 1.0 mg/ml of trypsin. Each bar represents the mean ± SD ($n = 3$). Statistical comparison was made by two-sided t-test ($\alpha = 0.05$) at each sampling point. *$P < 0.01$: significant difference versus TB without trypsin and TB with 0.01 mg/ml trypsin (from 1 to 14 days). **$P < 0.01$: significant difference versus TB without trypsin (from 3 to 14 days).

SEM observation showed thinning of the upper layer of SC film that was in direct contact with the enzyme solution (Fig. 5). Tokiwa and Calabia (2006) reported that the enzymatic degradation of aliphatic polyesters by hydrolysis is a two-step process. The first step is adsorption of the enzyme on the surface of the substrate via the surface-binding domain, and the second step is hydrolysis. It is likely that the significant decrease in SC weight during the first stage is caused by interaction between trypsin and SC previously exposed on the surface of the film. In our previous study, SC film implanted under the skin of rats degraded gradually over a period of 6 weeks or more (Nishida et al., 2011). The *in vivo* enzymatic degradation of SC may be less than the degradation occurring in 1.0 mg/ml of trypsin *in vitro*.

3.4. Drug release from SC film

3.4.1. FA release from SC film

The release profile of FA as a model protein drug from SC films (SC-FA and SC-GL-FA film in Table 1) was investigated in the absence and presence of trypsin. The release rate of FA in the absence of trypsin was 22% over 14 days (Fig. 6). We previously reported that FA, a charged protein model drug (*pI*: 4.7–4.9), was released for a longer period of time from SC film, gel, and sponge than fluorescein isothiocyanate–dextran, an uncharged model drug (Nishida et al., 2010, 2011). Although the *pI* of SC in solution is 5–6 (Capar et al., 2008), in the hydrated film state SC has a charged region of hydrophilic amino acids such as Lys and Arg between the β-sheet structure regions. It appears that FA release is suppressed by the ionic interactions between the hydrophilic region of SC and charged FA. On the other hand, FA release from SC films under enzymatic conditions was significantly faster than that in the absence of enzyme after 1 day in 1.0 mg trypsin, and after 3 days in 0.01 mg trypsin (Fig. 6). Forty-two percent and 92% of FA was released after 14 days in the presence of 0.01 and 1.0 mg/ml trypsin, showing that FA release increased with increasing concentrations of trypsin in the dissolution medium. Importantly, in dissolution medium containing 1.0 mg/ml trypsin, 62% of the FA was released after 3 days. Approximately 42% of the weight of the SC film remained after 3 days exposure to 1.0 mg/ml trypsin, indicating that 58% of SC was degraded (2% SC film in Fig. 4), consistent with the results of FA release. Because the hydrophilic region between the β-sheet regions of SC was enzymatically degraded, FA could be released

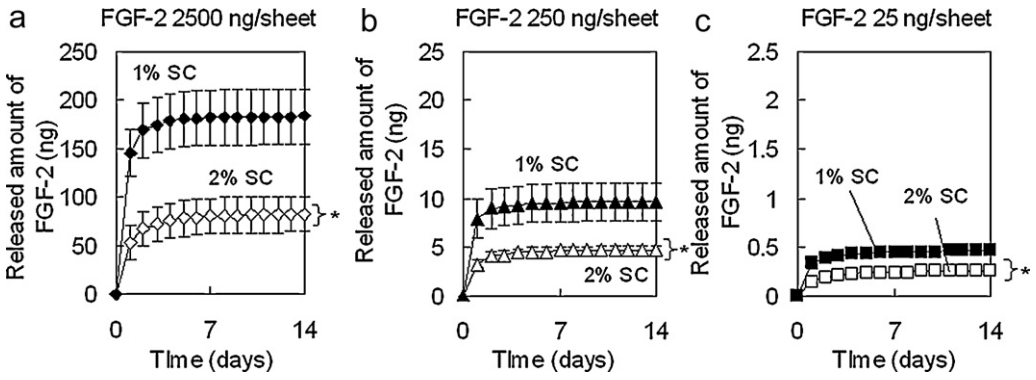

Fig. 7. *In vitro* release profiles of FGF-2 from 2% and 1% SC film incorporating 2500, 250, or 25 ng of FGF-2 (◊: 2% SC-FGF2500 film, Δ: 2% SC-FGF250 film and □: 2% SC-FGF25 film, ●: 1% SC-FGF2500 film, ▲: 1% SC-FGF250 film and ■: 1% SC-FGF25 film) in alpha minimum essential medium (αMEM) supplemented with 1% albumin and 1% penicillin–streptomycin at 37 °C. Each bar represents the mean ± SD ($n = 3$). Statistical comparison was made by two-sided t-test ($\alpha = 0.05$) at each sampling point. *$P < 0.01$: significant difference versus 1% SC (from 1 to 14 days).

rapidly from the matrix. Yang et al. (2007) investigated the release profiles of 10-hydroxycamptothecin (HCPT) encapsulated in bovine serum albumin nanoparticles (BSA NPs) in the absence and presence of trypsin. BSA NPs containing HCPT showed enhanced release upon trypsin exposure: ~25% release in 40 h in the absence of trypsin but ~90% release in 20 h in the presence of trypsin. The release of Rose Bengal as a model drug from human serum albumin nanoparticles is likely too slow to be practical, but the presence of trypsin accelerates the release rate of this encapsulated drug from nanoparticles (Lin et al., 2001). Wongpaint et al. (2010) investigated the release of ^{125}I-labeled FGF-2 from silk fibroin scaffolds in non-degradation and degradation conditions using protease type XIV from *Streptomyces griseus* (EC 3.4.24.31). In non-degradation conditions, FGF-2 immediately diffused from the scaffold and gradually plateaued at a release fraction of about 30% after 24 h. However, in the presence of degradative enzymes, the initial burst release was 75.7%, and then the FGF-2 in the scaffold was gradually released until 89.0% was released by day 7. In our study, FA was released very slowly by diffusion of FA from the SC matrix, but trypsin accelerated the release and hydrolyzed the SC film.

In our previous *in vivo* study, the degradation of SC film containing FA was evaluated by implanting the SC film under the skin of rats, and the amounts of remaining FA and the weight of the films were determined. FA release was sustained *in vivo* for more than 6 weeks. The amount of FA in the film decreased with the degradation of SC (Nishida et al., 2011). Thus, *in vivo* release of a protein drug from SC film may be slower than its release in *in vitro* conditions in the presence of 1.0 mg/ml of trypsin.

In this study, GL release from SC films was not evaluated. However, we predict that GL is released rapidly from SC film after immersion, because GL is a highly water-soluble agent, and in our preliminary studies, the weight of the SC film containing GL (dried film) decreased immediately after immersion in PBS. This weight loss was equal to the GL mass in the film, while remarkable weight loss of films with SC alone was not found after immersion (not shown). As for FA release from SC film with GL, the addition of a plasticizer, GL, did not change the profile of FA release from the film, regardless of whether trypsin was present (Fig. 6), suggesting that GL has an insignificant effect on the release of drugs such as FA.

3.4.2. FGF-2 release from SC film

Immobilization of growth factors via ionic interactions can be used to control their release. FGF-2 can be bound to gelatin hydrogels by poly-ion complexation and its release controlled (Tabata and Ikada, 1999). Wongpaint et al. (2010) suggested that FGF-2 release from silk fibroin scaffolds is sustained by ionic interactions between fibroin (*pI*: 3.8–4.5) and FGF-2 (*pI* 9.6). Furthermore, the release of epidermal growth factor from gelatin-based sponge, film, and ointment has also been investigated for wound healing (Okumura et al., 1990; Ulubayram et al., 2001).

As described above, amino acid analysis of SC (Takasu et al., 2002) has shown high amounts of serine (Ser, 33.2–39.0%), glycine (Gly, 14.1–16.0%) and aspartic acid/asparagine (Asp/Asn, 11.3–15.7%). Furthermore, SC has a hydrophilic region containing charged residues (Glu + Asp, and Lys + Arg) between the sequences constructing the β-sheet (Garel et al., 1997). The *pI* of SC is 5–6 in solution (Capar et al., 2008), so it is likely that FGF-2 (*pI*: 9.6) is immobilized on SC via ionic interactions. The release properties of FGF-2 from 2% and 1% SC films containing 2500, 250, or 25 ng of FGF-2 (2% and 1% SC-FGF2500, SC-FGF250, and SC-FGF25 in Table 1) were evaluated in dissolution medium in the absence of trypsin. FGF-2 was immediately released from 2% and 1% SC films, and the release gradually plateaued after 3 days (Fig. 7). Two percent SC films significantly exhibited a smaller burst release of FGF-2 than 1% SC films after 1 day. In the absence of trypsin, the release rate of a protein drug such as FGF-2 may be extremely low. The incorporated FGF-2 was released from acidic gelatin hydrogels within 1 day up to 30%, but thereafter no substantial release was observed in PBS (Tabata and Ikada, 1999). On the other hand, the radioactivity remaining in ^{125}I-labeled FGF-2-incorporating gelatin hydrogels also decreases gradually and correlates with the degradation of gelatin hydrogels incorporating FGF-2 after implantation into back subcutis of mice (Tabata et al., 1999). Wongpaint et al. (2010) reported that, in the absence of degradative enzymes, the release of ^{125}I-labeled FGF-2 from hexafluoroisopropanol (HFIP)-derived silk fibroin scaffolds is suppressed after the initial burst release (about 30% after 24 h), but the release increased to 89% in the presence of degradative enzymes after 7 days. In the current study, the release of FA from SC was accelerated in the presence of trypsin *in vitro* (Fig. 6), so it was expected that FGF-2 would also be released gradually as the SC film was degraded; however, this could not be verified experimentally as FGF-2 is not stable in solution in the presence of trypsin. As for resistance from enzymatic degradation of protein activity in the SC preparation, Zhang et al. (2006b) reported that the resistance to trypsin digestion of L-asparaginase (ASNase) bioconjugated to SC is greatly enhanced as compared with ASNase alone. The Michaelis constant (K_m) of SC-ASNase conjugate is 65 times lower than that of the ASNase alone, and insulin conjugated to SC also has a longer half-life (2.7 times more than non-conjugated insulin) in human serum (Zhang et al., 2006a). In our study, the SC film became thin depending on the concentration of trypsin in the incubation medium (Fig. 5), suggesting that enzymatic degradation of SC films occurred gradually on the

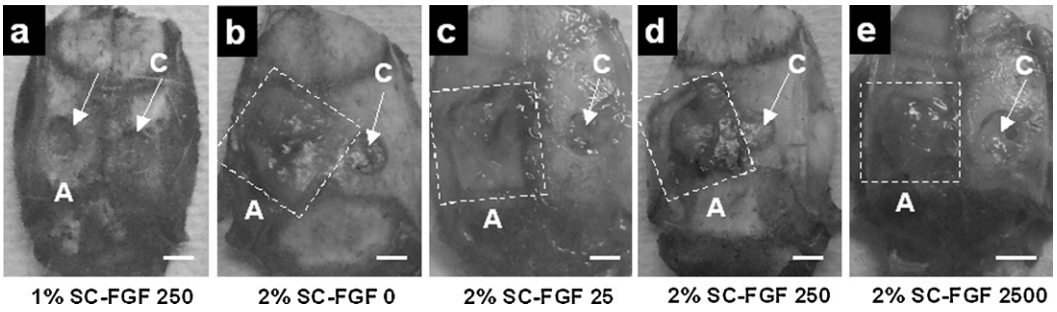

Fig. 8. Photographs of parietal bone defect treated with SC film (left, A) and not treated (right, C) after 2 weeks in rats: (a) 1% SC film incorporating 250 ng of FGF-2 (1% SC-FGF250) group (this film was not evident after 2 weeks); (b) 2% SC film without FGF-2 (2% SC-FGF0) group; (c) 2% SC film incorporating 25 ng of FGF-2 (2% SC-FGF25) group; (d) 2% SC film incorporating 250 ng of FGF-2 (2% SC-FGF250) group; (e) 2% SC film incorporating 2500 ng of FGF-2 (2%SC-FGF2500) group; dotted line, remaining SC films, tissue growth was observed with 2% SC-FGF2500 and 2% SC-FGF250 film (A in a and b); arrows, artificial defect hole (C in a–e and A in a).

surface of the film as discussed above. This suggests that SC has the potential to prevent the entry of enzymes into the preparation. We believe that enzymatic attack against proteinaceous drugs, such as FGF-2, is prevented more effectively by their incorporation into SC preparations than as individual proteins.

3.5. *In vivo study for the regeneration of bone defects treated with SC films incorporating FGF-2*

SC hydrogel sheets have been reported to have practical use as a dressing for wound healing (Tsubouchi, 1999; Teramoto et al., 2008). Recent reports on *in vivo* experiments showed that SC has no immunogenicity and thus can be used effectively in biomedical applications (Zhang et al., 2006a), and SC films show low inflammatory response as assayed by TNFα release (Dash et al., 2009). SC films prepared with PEG-diglycidyl ether show cytocompatibility (Xie et al., 2007), so that SC film can probably be used in biocompatible and biodegradable devices for drug delivery.

The incorporation of FGF-2 in biocompatible and biodegradable gels and scaffolds has been investigated as a way of avoiding the short half-life of FGF-2 and allowing for longer release *in vivo* (Tabata et al., 1999; Huang et al., 2008; Mabilleau et al., 2008; Takekawa et al., 2010). It was reported that the sustained release of FGF-2 from acidic gelatin hydrogel leads to prolonged vascularization (Tabata et al., 1999). Iwakura et al. (2003) reported that a gelatin sheet incorporating FGF-2 accelerated sternal healing after bilateral internal thoracic artery removal in normal and diabetic rats. In another study, a polylactic and polyglycolic acid copolymer, amalgamated with gelatin sponge, released incorporated FGF-2 and bone morphogenic protein (BMP). As a result, skull bone damage in rats was diminished by enhanced calcification due to the osteoinductive activity of BMP, which was enhanced by FGF-2 (Tanaka et al., 2006).

For *in vivo* studies, FGF-2 was incorporated in SC films containing GL, and 5 mm × 5 mm films were prepared (2% SC-FGF2500, 250, 25, 0, and 1% SC-FGF250; Table 1). Each SC film was implanted to cover a 3-mm-inner diameter hole drilled in the skulls of rats. Visual observation after 2 weeks showed that all of the 2% SC films covered the parietal bone defects intact (b–e in Fig. 8), but the 1% SC film was not present (a in Fig. 8). This suggested that the 1% SC film was degraded faster than the 2% SC film. This finding is best explained by the enzymatic degradation behavior of SC films *in vitro* (Fig. 2). Furthermore, 2% SC-FGF250 and 2500 films were markedly thickened due to tissue growth (d and e in Fig. 8). There were no obvious signs of inflammation at the site. In histological observations, larger amounts of collagen-like fiber tissue deposition were found, and the fiber tissue was more closely connected to the lip of the hole covered with SC film containing FGF-2 (2% SC-FGF25, e and f; 2%SC-FGF250, g and h; 2% SC-FGF2500, i and j in Fig. 9 and 2% SC-FGF250 in Fig. 10) than that in holes not covered with the film or covered with SC film not containing FGF-2 (Control, a and b; 2% SC-FGF0, c and d in Figs. 9 and 10). Although we found no

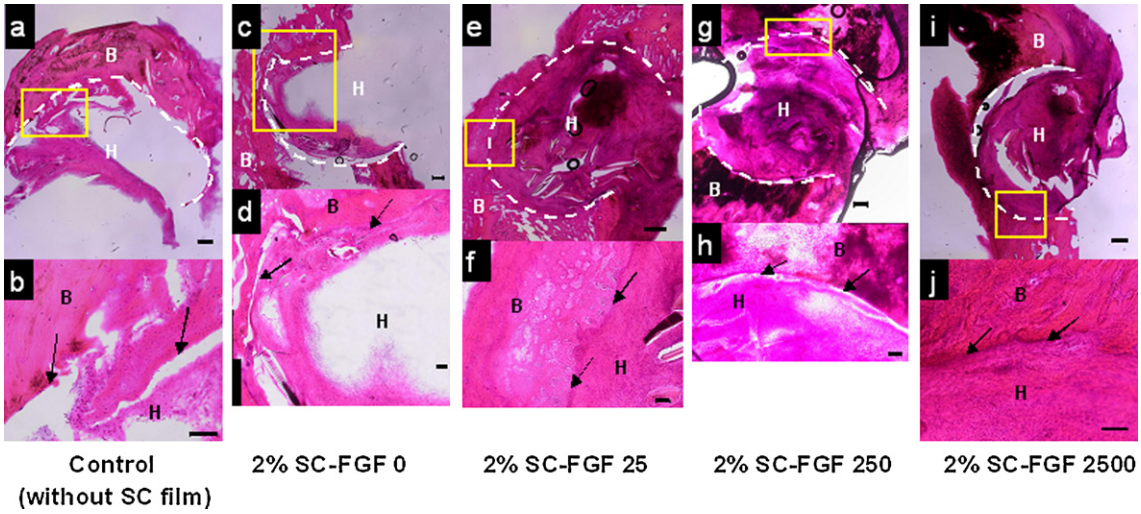

Fig. 9. Photographs of histological horizontal sections of parietal skull bone defects not treated (a and b) and treated with SC film (c–j) after 2 weeks in rats: (a) and (b) control group (not treated, SC film); (c and d) 2% SC film not incorporating FGF-2 (2%SC-FGF0) group; (e) and (f) 2% SC film incorporating 25 ng of FGF-2 (2%SC-FGF25) group; (g and h) 2% SC film incorporating 250 ng of FGF-2 (2%SC-FGF250) group; (i and j) 2% SC film incorporating 2500 ng of FGF-2 (2% SC-FGF2500) group. Dotted lines and arrows: the borders between the bone and the defected holes, (B) skull bone, (H) hole of skull bone defect; scale bar: (a, c, e, g, and i) 300 μm; (b, d, f, h, and j) 100 μm.

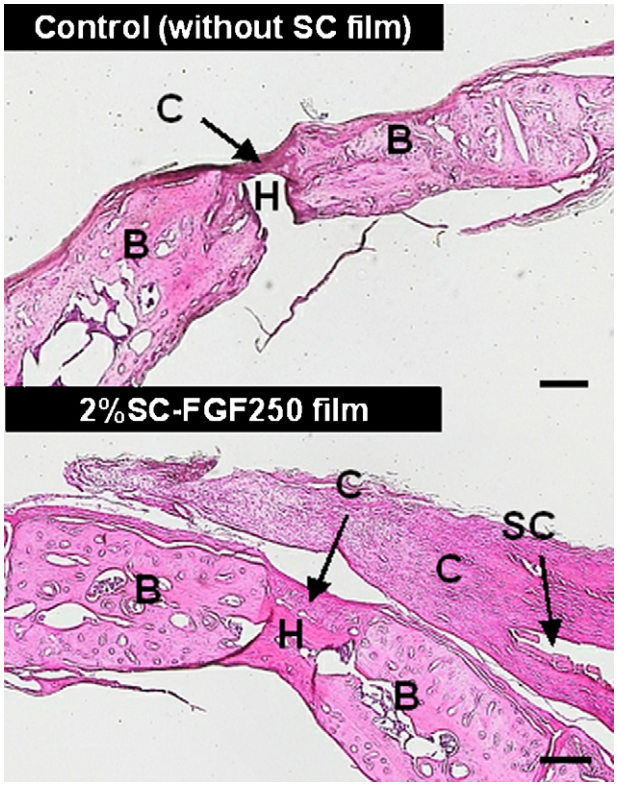

Fig. 10. Photographs of histological vertical sections of parietal skull bone defects treated with SC film containing 250 ng of FGF-2 (2%SC-FGF250 film) after 2 weeks in rats. (B) Skull bone, (H) hole of skull bone defect, (C) collagen-like fiber tissue, (SC) sericin film; Scale bar: 1 mm.

osteoid tissue formation in any of the holes, the implantation of SC film containing FGF-2 seemed to release FGF-2 gradually and to stimulate the growth of collagen-like fiber tissue. We believe that the growth of fiber tissue in defect holes, caused by the release of FGF-2 from SC film covering the holes, accelerates bone formation, because the collagen matrix works as a scaffold to promote the proliferation of mesenchymal stem cells, cartilage cells, and osteoid tissue formation (Reddi, 1981). In immunohistochemistry, alkaline phosphatase (ALP), osteocalcin, and tartrate-resistant acid phosphatase (TRAP) staining are used to stain osteoblasts, collagenous tissue, or osteoclasts (Taguchi et al., 2005). To make our findings more definitive, immunostaining will be necessary. In the current study, the administration of FGF-2 alone was not evaluated. But it is reported that FGF-2 solution did not enhance bone regeneration and neovasuclarization (Yamada et al., 1997; Ikada and Tabata, 1998) probably because of too short half-life period of FGF-2 in the body, and the administration of FGF-2 can be greatly improved by allowing sustained and localized release. We think that the combination of the SC film and FGF-2, and the prolonged localization of them at the site are effective. Thus, SC film may be useful in drug-releasing and biodegradable devices, and FGF-2 incorporated into film may accelerate bone remodeling and wound healing.

4. Conclusions

In the absence of trypsin, SC film swelled rapidly, kept its shape, and remained intact for 28 days or longer due to form β-sheet structures. In the presence of trypsin, SC film gradually degraded, with the rate of degradation depending on the concentration of trypsin, indicating that SC likely underwent hydrolysis and is thus biodegradable.

SC film incorporating a model protein drug, FA, also gradually degraded in the presence of trypsin, and released FA in a sustained manner for 2 weeks or longer. In the absence of trypsin, the release of FA was markedly slower.

To evaluate the practical applicability of SC film for regenerative medical therapy, such as inducing the repair of defective tissues, FGF-2 was incorporated in SC films and the films were implanted on skull wounds in rats for 2 weeks. The release of FGF-2 was suppressed in the absence of trypsin *in vitro*, suggesting that FGF-2 is trapped in SC film by ionic interactions. In the *in vivo* study, collagen-like fiber tissue grew abundantly around films incorporating 2500 or 250 ng of FGF-2 by 2 weeks after implantation.

Acknowledgments

The authors thank Dr. Hajime Tamura for his valuable advice on the histological observation, and Mr. Kiyohisa Ouchi for his helpful advice on this study.

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