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Sustained-release of protein from biodegradable sericin film, gel and sponge

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ABSTRACT

A silk protein, sericin, contains 18 kinds of amino acids, mostly polar side chains forming a complex of three principal polypeptides. The major polypeptides exhibit hydrophobic characteristics by forming a β-sheet structure in a hydrate state. As a drug-releasing biomaterial made by an aqueous process without using any cross linker, sericin is expected to form various hydrophobic dosage forms. However, its dosage form, with respect to the molecular weight and concentration of sericin, and its biodegradation behavior has not been studied in detail. In this study, the film, gel and sponge of sericin were prepared and examined to determine the release properties of the charged protein, fluorescein isothiocyanate–albumin (FA). The film and gel, as solid and semisolid forms, respectively, were also evaluated for their biodegradation behavior. For *in vitro* release, FA was sustained-released from these preparations. The concentration and dosage form markedly affected FA release. For *in vivo* biodegradation, the sericin preparations implanted subcutaneously in rats gradually decreased in size and weight. Histological examination indicated no marked inflammation at the site. As for *in vivo* release, FA remained for 3–6 weeks or more in rats. These findings suggest that sericin is suitable for use as a drug-releasing biomaterial.

1. Introduction

Fibroin and sericin (SC) comprise the silkworm (*Bombyx mori*) cocoon. SC composes 25–30% of the silkworm cocoon and covers the fibroin fibers like glue to form the cocoon. The SC protein contains 18 kinds of amino acids, mostly polar side chains such as hydroxyl, acidic and basic amino acids (Komatsu and Yamada, 1975), forming a complex of three principal polypeptides with molecular weights of 150, 180–250 and 400 kDa (Takasu et al., 2002; Teramoto et al., 2005). The amino acid analysis of SC (Takasu et al., 2002) showed that the amino acid compositions of all three SC 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). Correspondence of the amino acid composition of SC with these genes suggested that the 150- and 400-kDa SCs correspond to Ser1 proteins (77–331 kDa) encoded by the Ser1 gene, and that the 250-kDa SC corresponds to S-2 protein (227 kDa) encoded by the Src-2 gene (Takasu et al., 2002). The most abundant component is the largest SC (400 kDa), which corresponds to the Ser1C protein (331 kDa) (Takasu et al., 2002). A repetitive 38-amino acid sequence rich in Ser (40%) dominates a large part of the Ser1C protein and is predicted to have a strong tendency to form a hydrophobic 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 (227 kDa), has less β-sheet forming propensity and higher hydrophilicity than the 150- and 400-kDa polypeptides. This is because the 250-kDa SC contains larger amounts of β-sheet-breaking residues like Glu, glutamine (Gln) and Lys, and smaller amounts of β-sheet favoring residues like threonine (Thr) and tyrosine (Tyr) compared to the 150- and 400-kDa SCs (Takasu et al., 2002). Thus, SC has unique hydrophobic and hydrophilic characteristics, and it is expected to form hydrophobic matrices and bind a charged protein through its polar side chains.

Silk spinning and weaving are traditional trades in Japan that have contributed to the development of the field of sericulture. Through a silk scouring process involving an alkali treatment with high-pressure steam heating, SC is degraded into small molecular weight fragments and removed as a waste product. However, SC hydrolysate is reported to have various functions making it useful as a cell attachment enhancer (Minoura et al., 1995; Tsubouchi et al., 2005), moisturizer (Zhang, 2002), UV-resistant agent (Dash et al., 2008a), antioxidant agent (Kato et al., 1998; Dash et al., 2008b) and enzyme carrier (Zhang et al., 2004). Recently,

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a mammalian factor-free medium using SC hydrolysate has been developed with superior characteristics to commercial serum-free media for cell culture (Kobayashi et al., 2009). Miyamoto et al. (2009) also reported that SC (average molecular weight: 30 kDa) is useful for cryopreservation of human adipose tissue-derived stem/progenitor cells. These reports suggest SC is suitable as a biomaterial for medical purposes. As for dosage forms of SC hydrolysate as a biomaterial, the recent film and sponge-like scaffolds of SC/gelatin and SC/polyvinyl alcohol (PVA) using crosslinkers have been shown to exhibit high physical strength and to gradually degrade in phosphate buffered saline (PBS) or water over several weeks (Mandal et al., 2009; Aramwit et al., 2010). Indeed, these films and scaffolds have been found to have sufficient biocompatibility as they cause limited inflammation (Dash et al., 2009; Mandal et al., 2009). Moreover, previous studies reported improved oxygen permeability by increasing the SC content in a film comprising PVA (Wang et al., 1998) and effective induction of hydroxyapatite nucleation by a SC film in a biomimetic solution (Takeuchi et al., 2005). A SC cream was also reported to have practical use as a dressing for wound healing (Aramwit and Sangcakul, 2007). Kundu et al. (2008) reviewed the potential for biomedical and biotechnological applications of SC and summarized that for protein and drug delivery, SC hydrolysate can be conjugated with L-asparaginase, β-glucosidase and insulin using a crosslinker through its surface-active groups (i.e., –OH, –COOH, and $–NH_2$). The success of SC utilization lies in its hydrophilic nature with lower antigenicity and its immunogenic properties, in addition to its higher half-life *in vivo* due to filtration by the kidney allowing increase retention.

Intact or low degraded SC containing high molecular weight fragments (150–400 kDa) is obtained by dissolving it in lithium bromide (LiBr) solution or heating for tens of minutes in water (Teramoto et al., 2005; Mase et al., 2006; Komatsu, 1975). It is available in various forms prepared by aqueous processes without a crosslinker. Kundu et al. (2008) used intact SC to prepare stable matrices, including hydrogels, porous materials, and films, with good properties. Other groups have prepared a film from a SC aqueous solution by the casting method (Teramoto and Miyazawa, 2005; Teramoto et al., 2008), a moldable gel through an aging process of the SC solution (Zhu et al., 1995; Teramoto et al., 2005), a hydrogel sheet for use as a dressing for wound healing (Tsubouchi, 1999; Teramoto et al., 2008), and a porous sponge by freeze-drying (Tao et al., 2005).

Recent studies using transgenic silkworm strains have successfully expressed recombinant proteins, enhanced green fluorescent protein and human serum albumin, secreted in their SC layer (Ogawa et al., 2007; Tomita et al., 2007), and a cell attachment factor in the cocoon for application in the development of a biocompatible and biodegradable artificial blood vessel (Takabayashi, 2009). As research on SC is progressing, a new direction of sericulture is expected.

As SC shows potential as a biomaterial for drug release, its dosage forms, regarding the molecular weight and concentration of SC, have not been studied in detail. For a drug delivery system, several biocompatible and biodegradable *drug releasing* materials have already been developed. Some materials, including synthetic polymers like poly(lactic-co-glycolic acid), natural polymers such as gelatin and chitosan, and their combinations (Okada et al., 1994; Kushibiki et al., 2006; Kanazawa et al., 2009; Bhattarai et al., 2010; Takekawa et al., 2010), are already in use for medical purposes. By poly-ion complexation, gelatin hydrogels bind fibroblast growth factor (Tabata and Ikada, 1999) and cationized gelatin hydrogels bind plasmid DNA and epidermal growth factor (Kushibiki et al., 2006; Hori et al., 2007). These gels sustained protein drug release until degradation of the gelatins themselves. Kang and Song (2008) reported that FA (p*I*: 4.7–4.9), when negatively charged, easily binds to the amino groups in chitosan, allowing sustained release from a modified chitosan hydrogel for 1–2 weeks. The ionic interaction between a charged protein drug and charged drug releasing materials is useful for a drug delivery system. Although such materials have excellent characteristics as biomaterials, their applications have been limited due to low compatibility with certain drugs, use of large amounts of organic solvents or toxic cross-linker, and problems arising from bovine spongiform encephalopathy. Therefore, development of better options for *drug releasing* materials with sufficient biocompatibility is important.

In this study, we examined the film, gel and sponge dosage forms of SC containing high molecular fragments as a *drug releasing* material for potential use as a medical dressing film, ointment-based injectable gel, and implant, respectively. We measured *in vitro* the release properties of a charged model protein drug, fluorescein isothiocyanate–albumin (FA), for each dosage form using different molecular weights and concentrations of SC. We also examined *in vivo* the biodegradation rate of SC preparations and the release of FA implanted subcutaneously in rats.

2. Materials and methods

2.1. Animals

Seven-week-old male Sprague–Dawley rats were purchased from Charles River Japan Co., Ltd. (Kanagawa, Japan). The animal experiments in this study were conducted in accordance with the recommendations of the Kissei Pharmaceutical Animal Care and Use Committee and with the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society.

2.2. 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 is 98%, Kougensha Co., Ltd., Nagano, Japan) (Mase et al., 2006). FA (66 kDa, Sigma–Aldrich Japan Co., Ltd., Tokyo, Japan) was used as the charged model drug. D-Sorbitol (Mitsubishi-Kagaku Foods Co., Ltd., Tokyo, Japan) was used as an additive to the SC films.

2.3. Preparation of SC solution, film, gel and sponge for the in vitro study

Table 1 shows the formulations of SC films, gels and sponges and preparation conditions of the SC solutions. For preparation of the 1% SC solutions, SC was dissolved in purified water (1:100, w/v) under various conditions: 100 °C for 20 min (SL-1A), 100 °C for 60 min (SL-1B) and 120 °C for 20 min (SL-1C). To dissolve SC at 120 °C, an autoclave (HA300M5, Hirayama Manufacturing Co., Saitama, Japan) was used. For preparation of the 2% SC solution, SC was dissolved in purified water (2:100, w/v) at 100 °C for 20 min (SL-2A). As the SC solutions included a portion of insoluble SC and other components, each solution was centrifuged at 3,000 rpm for 5 min to remove insoluble components, and then cooled to room temperature.

For *in vitro* SC preparations, FA (0.5 mg/ml) was dissolved in each SC solution, and then 2 ml SC solution containing 1.0 mg FA was dispensed into test tubes (23-mm inner diameter). Films (F-1A, F-1B, F-1C and F-2A) were obtained by drying each solution (SL-1A, SL-1B, SL-1C and SL-2A) in the tubes at 40 °C for 1 day. Gels (G-1A, G-1B, G-1C and G-2A) were obtained by cooling each solution in the tubes at 5 °C for 1 day, and were conditioned at room temperature before use. Sponges (S-1A, S-1B, S-1C and S-2A) were obtained by freezing each solution in the tubes at −80 °C for 1 day and then thawing at room temperature for about 30 min just before use. The

Table 1
Formulations of SC preparation for *in vitro* study.

Formulations		Materials			Dissolution condition of SL		
		FA (mg/unit)	SL (ml/unit)		SL[a] (v/w ratio)	Temperature (°C)	Heating time (min)
Film	F-1A	1	2000[b]	SL-1A	1	100	20
	F-1B	1	2000[b]	SL-1B	1	100	60
	F-1C	1	2000[b]	SL-1C	1	120	20
	F-2A	1	2000[b]	SL-2A	2	100	20
Gel	G-1A	1	2000	SL-1A	1	100	20
	G-1B	1	2000	SL-1B	1	100	60
	G-1C	1	2000	SL-1C	1	120	20
	G-2A	1	2000	SL-2A	2	100	20
Sponge	S-1A	1	2000	SL-1A	1	100	20
	S-1B	1	2000	SL-1B	1	100	60
	S-1C	1	2000	SL-1C	1	120	20
	S-2A	1	2000	SL-2A	2	100	20

SC: sericin; FA: flurosecein isothicyanate–albumine; SL: SC solution.
[a] Concentration of the SC powder loaded in water.
[b] The water in the SC films was removed by the drying process.

frozen SC solution is insolubilized by allowing ice nuclei to grow during the freezing process thereby dehydrating the SC phase (Tao et al., 2005).

2.4. Molecular weight and concentration of SC solution

The molecular weight of SC dissolved under various heating conditions (SL-1A, SL-1B and SL-1C) was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The SC solutions were electrophoresed on 10% polyacrylamide gels (C10L, ATTO Corporation, Tokyo, Japan) and the proteins were stained with Coomassie Brilliant Blue R-250. The concentration of dissolved SC was calculated from the solid content weight of SC after drying at 40 °C.

2.5. In vitro release properties of FA from SC preparations

To evaluate the release rate of FA from the SC film (F-1A, F-1B, F-1C and F-2A), gel (G-1A, G-1B, G-1C and G-2A) and sponge (S-1A, S-1B, S-1C and S-2A), 5 ml PBS (pH 7.4) was added as the dissolution medium to each SC preparation in a test tube and incubated at 37 °C.

The tested medium was centrifuged at 3,000 rpm for 10 min to remove insoluble components, including disintegrated pieces of SC preparation, and the release rate of FA was measured over time by UV absorption spectrophotometry at 495 nm (UV-2500PC; Shimadzu Co., Kyoto, Japan).

2.6. Preparation of SC film and gel for in vivo study

For *in vivo* film and gel preparations, FA (0.5 mg/ml) was dissolved in 1% (SL-1A) or 2% (SL-2A) SC solution as described above. The film and gel were prepared with sterile purified water and aseptic containers at a clean bench.

To improve the flexibility of the films for practical use, D-sorbitol was added to 2% SC solution (SL-2A) (1:400, w/v). For film preparation, SL-2A (40 ml) with and without FA were cast on polyethylene dishes (10 cm × 10 cm) and dried at 25 °C with 30% relative humidity for 1 day. For implantation under the skin of rats, the films were cut into 1 cm × 1 cm square sheets.

For gel preparation, 1% (SL-1A) and 2% (SL-2A) SC solution (0.5 ml) with and without FA were added to disposable polypropylene syringes and cooled at 5 °C for 1 day to allow for gelation. Gels containing FA were conditioned at room temperature before use.

2.7. Animal operation

Rats were anesthetized by intraperitoneal administration of sodium pentobarbital (50 mg/kg), and received a full-thickness incision in the skin at a site on the back. Film sheets were implanted under the skin through the incision, and then the incision was closed with sutures. Gels (0.5 ml) were injected under the rat skin at the same site under ether anesthesia.

Rats were euthanized 1–6 weeks after drug delivery by an overdose of sodium pentobarbital. To evaluate the biodegradation rate of SC and the release properties of FA from the SC preparations, the implanted films or injected gels containing FA were carefully resected and washed in PBS. The resected SC films were dried for 24 h before analysis.

For histological observation, the SC films and gels without FA were removed with the surrounding tissue (including the skin, connective tissue and skeletal muscle) and fixed in 10% phosphate-buffered formalin. Paraffin-embedded sections were routinely stained with hematoxylin and eosin.

2.8. Bioerosion behavior of SC film and gel

Bioerosion of the SC film and gels *containing* FA was measured based on the change in weight after resectioning and drying (film only). The morphological changes of the SC film and gels *without FA* and histological inflammatory state of the tissues surrounding them were observed by microscopy.

2.9. Remaining amount of FA in SC film and gel used in vivo

The resected SC film and gels after implantation were dissolved in 8 M LiBr aqueous solution to dissolve SC. These LiBr solutions were centrifuged at 6,000 rpm for 10 min to remove insoluble components, including tissue from the rat. To determine the remaining amount of FA in the samples, fluorescence absorbance of FA (Ex 493 nm, Em 515 nm) was measured by fluorescence spectrophotometry (Spectra Max, Molecular Devices, Sunnyvale, CA, USA).

3. Results and discussion

3.1. In vitro study

3.1.1. Molecular weight and solubility of SC

SC was dissolved in purified water under various temperature and heating conditions (Table 1). Fig. 1 shows the molecular weights of SC by SDS-PAGE. Two bands, one at 250 kDa and a larger

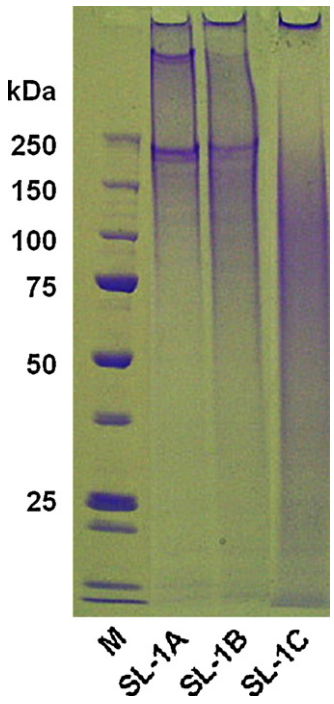

Fig. 1. SDS-PAGE patterns of SC dissolved under various dissolution conditions: SL-1A, 100 °C for 20 min; SL-1B, 100 °C for 60 min; SL-1C, 120 °C for 20 min; M: molecular weight marker.

one, were mainly detected in the dissolution conditions of 100 °C for 20 min (SL-1A) and 100 °C for 60 min (SL-1B); a smeared band around 100 kDa was detected in the condition of 120 °C for 20 min (SL-1C). The solubility of SC components degraded by the heat load increased with temperature and time in the order of SL-1A (73%) < SL-1B (86%) < SL-1C (96%); the concentration of SC had no notable influence on solubility (SL-1A: 73%; SL-2A: 76%). In intact SC dissolved in LiBr aqueous solution, three major molecular weight bands have been reported: 150, 180–250 and 400 kDa (Takasu et al., 2002; Teramoto et al., 2005). These bands were confirmed in intact SC dissolved in the same solution in our preliminary study (data not shown). The two bands seen in the SL-1A and SL-1B solutions were estimated to correspond to the 180-, 250- and 400-kDa bands of intact SC. As heating is reported to hydrolytically degrade SC (Teramoto et al., 2007), the size of SC most likely decreased, thereby improving its solubility as the heat load increased during dissolution of SC. All four SC solutions were used in subsequent experiments of SC films, gels and sponges.

3.1.2. *In vitro release profiles of FA from SC preparations*

In the aqueous extraction of SC, heating affects the molecular weight of the extracted SC (Mase et al., 2006). Generally, the molecular weight of the polymer and physical strength of the preparation are factors that influence drug release rate. The release of FA, used as the model drug in this study, from three SC dosage forms, film, gel and sponge, was examined using various molecular sizes of SC. Fig. 2 shows the appearance of each SC preparation. Although the SC gel and sponge tended to disintegrate with the increase of the heat load (Fig. 2), each SC preparation sustained FA release for up to 1 week. The FA release rates did not significantly differ relative to the molecular size of SC. All SC preparations tended to disintegrate and erode in PBS solution.

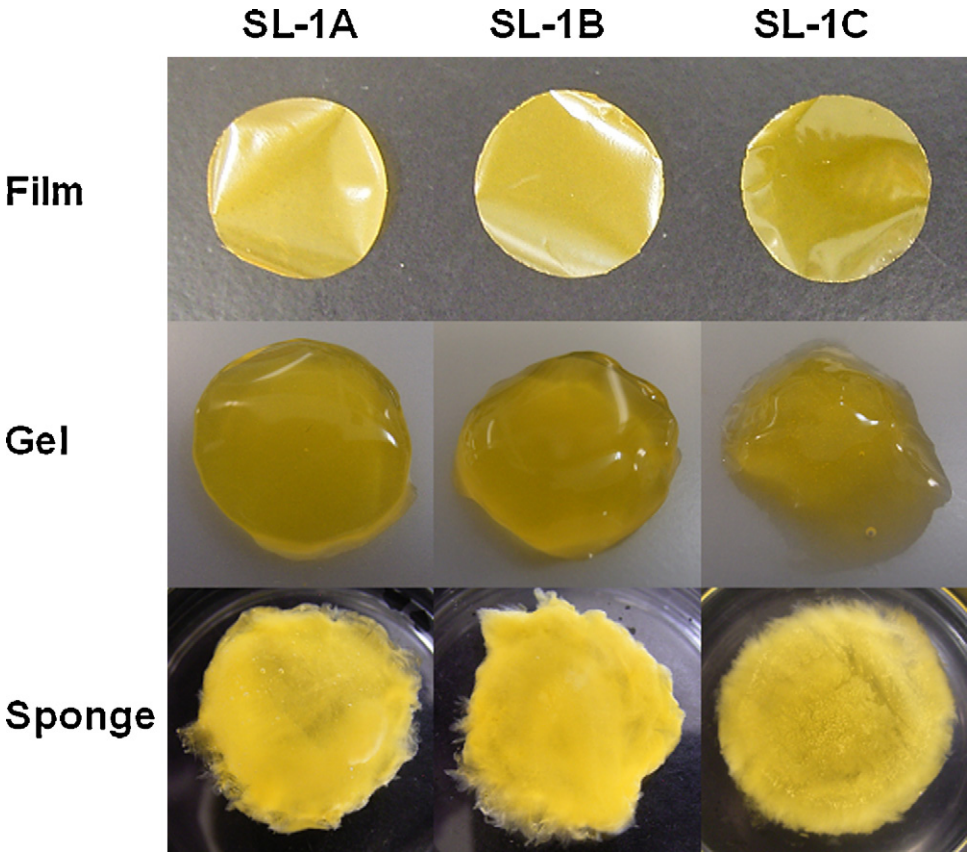

Fig. 2. Photographs of SC film, gel and sponge comprised of various SC solutions (SL-1A, SL-1B and SL-1C). Heating condition of SL-1A: 100 °C for 20 min; SL-1B: 100 °C for 60 min; SL-1C: 120 °C for 20 min. Scale bar: 10 mm. FA: fluorescein isothiocyanate–albumin.

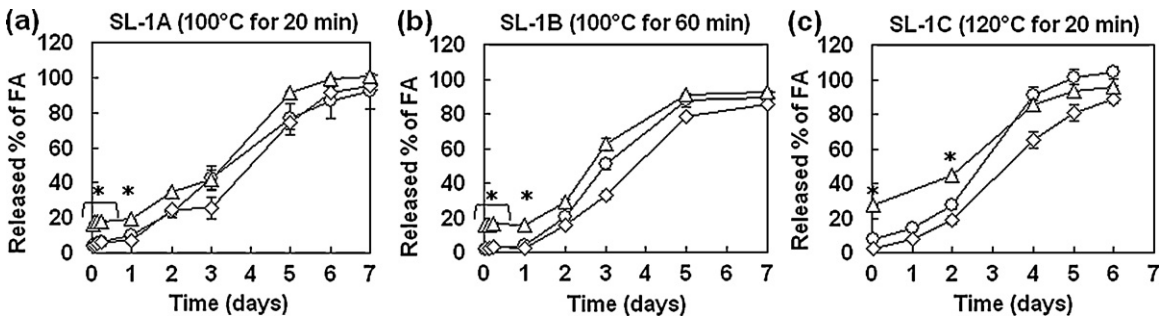

Fig. 3. *In vitro* release profiles of FA from various SC preparations comprised of SL-1A (a), SL-1B (b) and SL-1C (c) in PBS (pH 7.4) at 37 °C: (○) film; (◊) gel; (△) sponge. Each bar represents the mean ± SD ($n = 3$). Statistical comparison was made by two-sided *t*-test ($\alpha = 0.05$). $^{*}P < 0.01$: significant difference versus film and gel.

Drug delivery systems can utilize *ionic* interactions between charged protein drugs and charged sustained release materials. Sustained protein drug release has already been achieved with poly-ion-complexed gelatin hydrogels (Tabata and Ikada, 1999) and cationized gelatin hydrogels (Kushibiki et al., 2006; Hori et al., 2007), as well as with a negatively charged FA-bound chitosan hydrogel. Amino acid composition analysis of SC by the PTC method showed that SC contains 18 different amino acids, of which about 70% are polar amino acids. Of these, about 40% are neutral amino acids, about 20% are acidic amino acids, and about 10% are basic amino acids (Takasu et al., 2002). Two-dimensional electrophoresis indicates that the p*I* of SC in solution is between 5 and 6 (Capar et al., 2008), suggesting that SC is negatively charged at pH 7.4. However, the secondary structure of SC changes from a hydrophilic random coil conformation in solution to a hydrophobic β-sheet structure in a gel or moistened film (Teramoto et al., 2005). Structural analysis of the Ser1 gene coding major SC proteins (S1 proteins) indicated that S1 proteins consist of repetitive subunits of 8 peptides. The large peptides (peptides 6 and 8) are rich in Ser (40%) and form hydrophobic β-sheet structures through hydrogen bonds between the OH groups of the Ser and Thr side chains and the C=O groups of the backbone on the adjacent strands (Garel et al., 1997; Teramoto et al., 2007). On the other hand, SC has a hydrophilic region (peptide 3) containing charged residues (Glu + Asp = 13%, and Lys + Arg = 15%) between the hydrophobic sequence and the β-sheet (Garel et al., 1997). In this study, because SC was in the solid state as a gel, sponge and film in PBS (pH 7.4), the SC preparations were expected to form β-sheet structures and to contain charged residues in the hydrophilic region, analogous to peptide 3. Thus, forming a hydrophobic β-sheet structure changes the ratio of effective charged amino acid sequences. We therefore speculated that negatively charged FA interacts ionically with positively charged residues like Lys (p*I*: 9.75) and Arg (p*I*: 10.76) in the SC preparations in PBS (pH 7.4), thereby sustaining long release of FA. In our recent study, we examined fluorescein isothiocyanate–dextran (FD) with different molecular weights (FD4: 4 kDa; FD70: 70 kDa) as a non-charged model drug and FA (66 kDa) as a charged model drug (Nishida et al., 2010). The order of their release rates was FD4 < FD70 < FA, suggesting that SC can be used as a drug releasing material for high molecular weight drugs. Furthermore, if the drug is charged, its release can be sustained for an extended time.

As for differences in the dosage forms and concentrations of SC, sponges (1% SC) had a significant initial burst release of FA (Fig. 3), which was considered to be the result of small amounts of FA that leaked from the sponge during the thawing process. SC/gelatin sponge-like scaffolds with larger sized pores exhibit faster degradation than those with smaller pores (Mandal et al., 2009). In our previous study using scanning electron microscopy, an SC film did not have any pores on its surface, but the freeze-dried SC sponge had many pores on the thin membrane (Nishida et al., 2010). The available surface area of the SC sponge is thus expected to be larger than that of the SC film. In the SC gel, SC adopts a β-sheet structure that constructs the uniformly dense network structure of hydrogel (Garel et al., 1997; Teramoto et al., 2005). Taken together, FA is considered to diffuse more easily from the SC sponge. Fig. 4 shows the release profiles of FA from each SC preparation at the two different concentrations of SC (1% and 2%). To examine 2% SC preparations, we selected a typical dissolution condition of SC, 100 °C for 20 min, which allowed the least degradation of SC and most moldability of the preparation, thereby providing the slowest drug release. For 2% SC preparations, all dosage forms sustained FA release for 2 weeks or more, which was markedly longer than the stained release by the 1% SC preparations. These results suggest that the release of FA could be controlled by adjusting the concentration of SC. In addition, the initial burst release of FA from sponge was not observed in 2% SC preparations, compared to the 1% SC preparations (Fig. 4c). A previous study characterized the release mechanism of a FA/chitosan complex system via ionic charges, first in terms of the initial burst release and then by the degradation of the hydrogel (Kang and Song, 2008). The initial burst release was

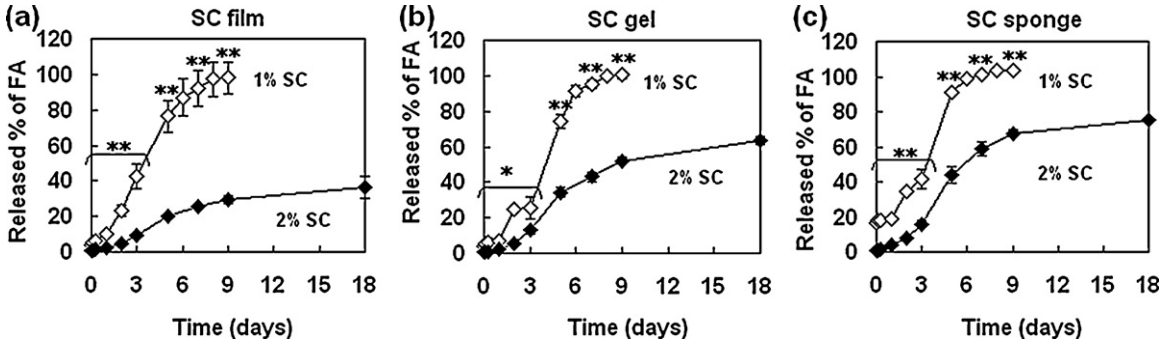

Fig. 4. *In vitro* release profiles of FA from the SC film (a), gel (b), or sponge (c) comprised of 1% (SL-1A, ◊) and 2% (SL-2A, ♦) SC solution in PBS (pH 7.4) at 37 °C. Each bar represents the mean ± SD ($n = 3$). Statistical comparison was made by two-sided *t*-test ($\alpha = 0.05$). $^{**}P < 0.01$, $^{*}P < 0.05$: significant difference versus 2%.

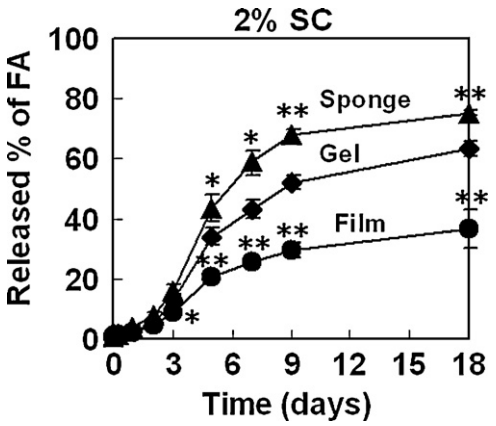

Fig. 5. *In vitro* release profiles of FA from various SC preparations comprised of 2% SC solution (SL-2A) in PBS (pH 7.4) at 37 °C: (●) film; (♦) gel; (▲) sponge. Each bar represents the mean ± SD ($n = 3$). Statistical comparison was made by two-sided t-test ($\alpha = 0.05$). $^{**}P < 0.01$, $^{*}P < 0.05$: significant difference versus gel.

attributed to free protein that did not participate in complex formulation. Moreover, the initial burst of the protein drug from an ion-complex system using gelatin was markedly suppressed by the increase in the charged carrier material ratio (Tabata et al., 1999; Hori et al., 2007). For the SC film, gel and sponge in this study, FA appears to ionically interact with SC more reliably and accurately with the increase in SC content.

The FA release rate from 2% SC preparations was the slowest for the film, followed by the gel and then sponge (Fig. 5). As mentioned above, the film had the most compact structure due to the drying process, compared with the gel which had a network structure of hydrogel containing water and the sponge which had a porous structure. FA in the film is therefore most likely trapped tightly within the SC structure. Based on these observations, SC appears to be a potential biomaterial for sustained release of charged protein drugs for three dosage forms, film, gel and sponge.

3.2. In vivo study

3.2.1. Bioerosion behavior of SC preparations

Aramwit et al. (2010) reported that the non-crosslinked SC hydrolysate/PVA scaffold completely dissolved in less than 30 min, while a high concentration of the crosslinker, genipin, inhibited the dissolution of SC in PBS. Utilizing the hydrophilic properties of SC hydrolysate, the crosslinked SC hydrolysate/gelatin scaffold could be degraded gradually for 28 days or more (Mandal et al., 2009). The intact or low degraded SC exhibits hydrophobic properties in the hydrate state due to formation of the β-sheet structure (Garel et al., 1997), resulting in a slower degradation rate. Degradation of intact SC is reportedly caused by hydrolysis of its hydrophilic region (Teramoto et al., 2007). Macrophages and peptidase are known causes of hydrolysis (Makino et al., 1985; Freddi et al., 2003). To evaluate the degradation of SC containing high molecular weight fragments, 250 kDa and 400 kDa, the film as the solid dosage form and the gel as the semisolid form were prepared in the same manner as the *in vitro* study. Following implantation of the film and injection of the gels under the skin of rats, the size of both dosage forms decreased over time (Fig. 6), with the film degrading slower than the gels (Fig. 7). At the first stage (week 1) of biodegradation, the film remained almost unchanged in shape (Fig. 6a, week 1) and weight (Fig. 7, F), while the gels rapidly reduced in size (Fig. 6b and c) and weight (Fig. 7, G1 and G2). These observations indicate that the gel was dehydrated at the first stage, as its formulation contained more than 98% water. Takeoka et al. (2004) also reported that the interperitoneal injection of SC solution into a rat model was dehydrated and thus degraded into small particles. As for the effect of SC concentration, the remaining weight of the gel in the 2% SC preparation tended to be larger than that in the 1% SC preparation (Fig. 7, G1 and G2). Thus, the rate of biodegradation of SC was affected by the dosage form and concentration of SC. Mandal et al. also reported differences in the degradation rates of a crosslinked SC hydrolysate/gelatin two-dimensional film and three-dimensional scaffold due to differences in the mechani-

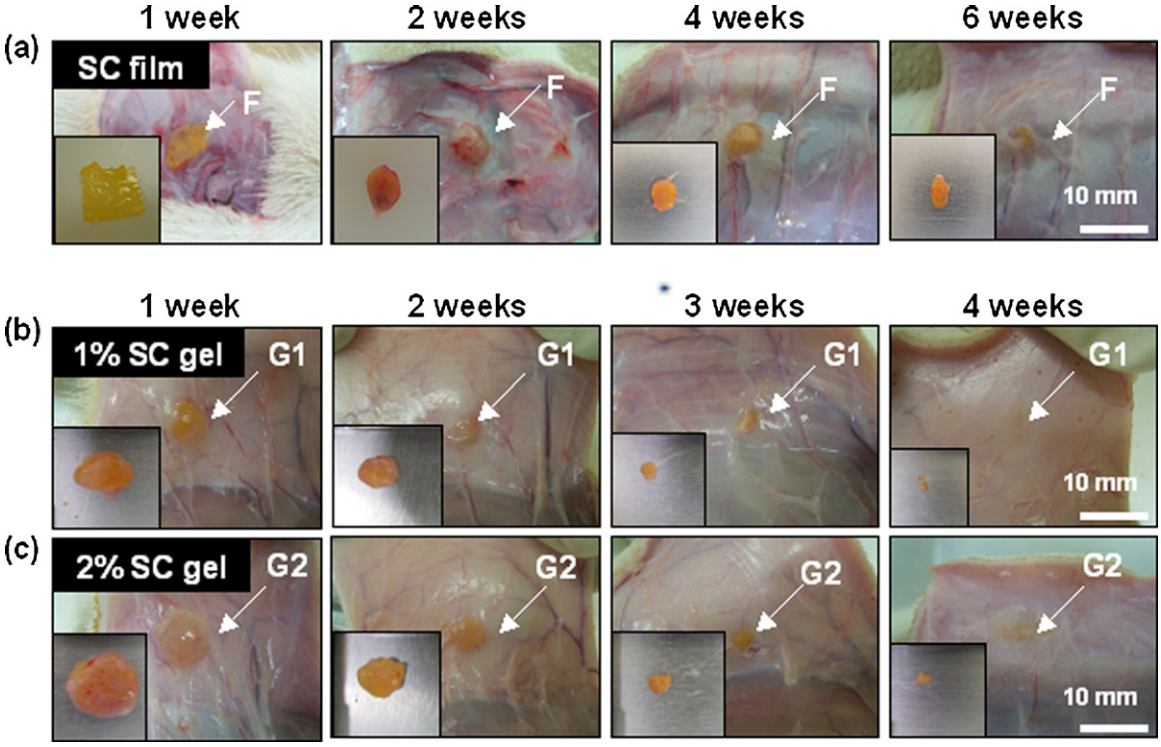

Fig. 6. Photographs of SC film (a) and gel (b: 1% SC; c: 2% SC) implanted subcutaneously in rats: F, SC film; G1, 1% gel; G2, 2% SC gel. Inserts show the SC preparations isolated from the connective tissue. Scale bar: 10 mm.

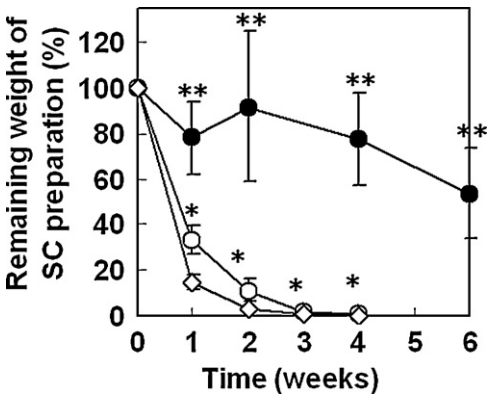

Fig. 7. Bioerosion profiles of the SC film and gel implanted subcutaneously in rats: (●) SC film (F); (◊) 1% SC gel (G1); (○) 2% SC gel (G2). Each bar represents the mean ± SD ($n = 5$). Statistical comparison was made by two-sided t-test ($\alpha = 0.05$). $^{**}P < 0.01$: significant difference versus G2, $^{*}P < 0.05$: significant difference versus G1.

cal integrity of the two forms of matrices, in addition to the available surface area in contact with the aqueous medium. They concluded that the films were more fragile and more susceptible to degradation than the scaffolds, although they found the same amount of degradation (about 40%) after 28 days for both film and scaffold containing SC/gelatin (5:5, wt%). In our *in vitro* study, FA release was sustained longer in the SC film than in the SC sponge and gel (Fig. 5). Moreover, the SC sponge was found to be easily disintegrated in PBS solution. Immersion of the larger available surface area of the SC sponge may thus accelerate hydrolysis of the high molecular weight SC.

On histological observation, local thickening of connective tissue was observed around the SC preparations at a relatively early stage, which decreased in size as degradation progressed (Figs. 6 and 8). A slight increase in capillary blood vessels was also observed around the connective tissue surrounding the SC preparations in some sections (Fig. 8a and b, at 2 weeks). This phenomenon indicates a kind of bio-defense system against the degraded foreign material (Okada and Toguchi, 1995). All SC preparations were encapsulated in a collagen-like material from connective tissue; the film was disintegrated into pieces (Fig. 8a), and the gel formed into a rigid mass (Fig. 8b and c). SC has been shown to be useful in mammalian factor-free medium for cell culture (Kobayashi et al., 2009) and for cryopreservation of human adipose tissue-derived stem/progenitor cells (Miyamoto et al., 2009), further suggesting its good biocompatibility. Indeed, Dash et al. (2009) and Mandal et al. (2009) have recently confirmed the cytocompatibility of SC film and SC/gelatin matrices in feline fibroblasts which showed normal spreading and proliferation without any cell cycle arrests. Low immunogenicity of such matrices as observed through tumor necrosis factor α release also revealed their potential as future biopolymeric graft materials. Similar to these recent studies, no marked inflammation was observed during biodegradation of SC in this study (Figs. 6 and 8). As a result, all three SC dosage forms appear to be biocompatible and biodegradable, suggesting their applicability in the implantation or injection of topical medicine.

3.2.2. *In vivo* profiles of FA release from SC preparations

After, respectively, implanting and injecting the SC film and gel under the skin of rats, remaining amounts of FA in the preparations were determined. The amount of FA in the film decreased with the degradation of SC (Fig. 9, F). In the gel, although its weight rapidly decreased at the first stage of biodegradation (Fig. 7, G1 and G2), the decrease in FA was slow (Fig. 9, G1 and G2). These results suggest that SC trapped FA, preventing dehydration of the gel and diffusion of FA at the first stage biodegradation.

The remaining amount of FA in the film was greater than that in the gel, confirming the *in vitro* release results (Fig. 5). FA was sustained *in vivo* for more than 6 weeks in the film and for up to 3

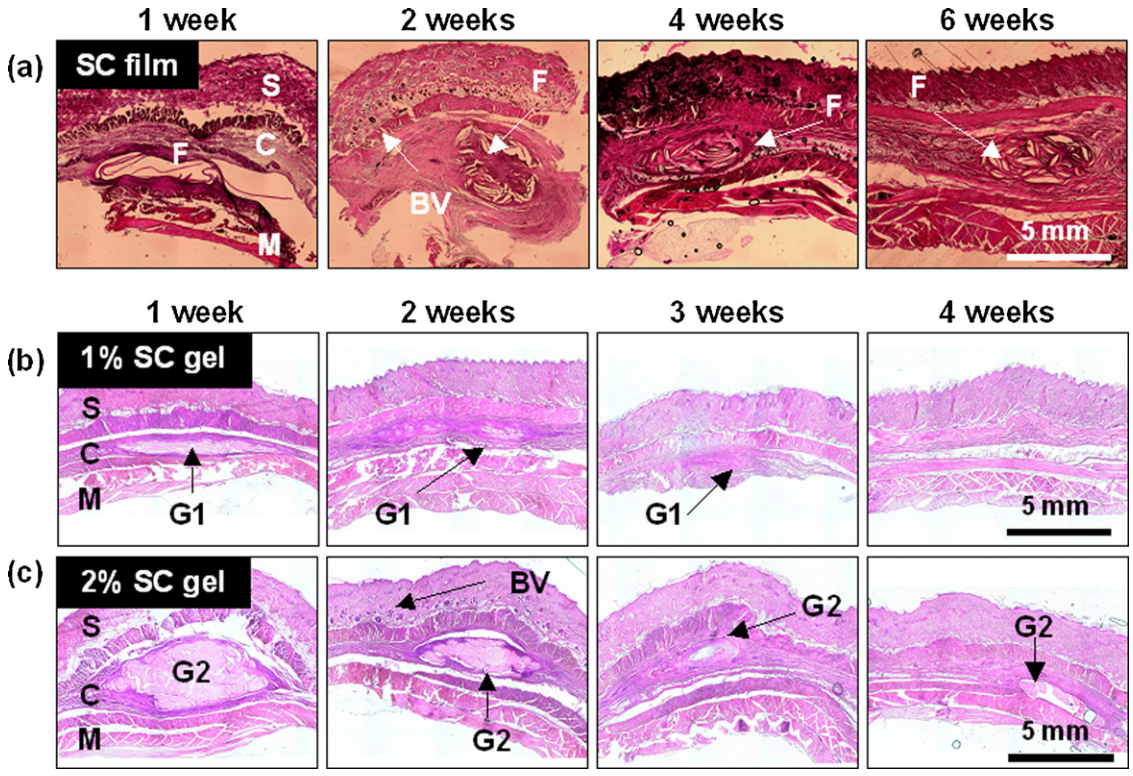

Fig. 8. Histological observations of the SC film (a) and gel (b: 1% SC; c: 2% SC), and the tissues after subcutaneous implantation in rats. In the section at 4 weeks, 1% SC gel (b) could not be observed clearly. F, SC film; G1, 1% SC gel; G2, 2% SC gel; S, skin; C, connective tissue; M, skeletal muscle; BV, capillary blood vessel. Scale bar: 5 mm.

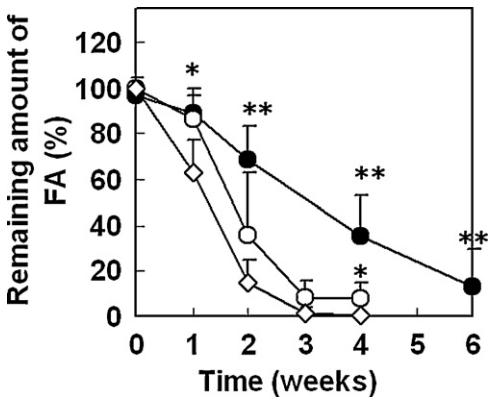

Fig. 9. Remaining amount of FA in the SC film and gel after subcutaneous implantation in rats: (●) SC film (F); (◊) 1% SC gel (G1); (○) 2% SC gel (G2). Each bar represents the mean ± SD ($n = 5$). Statistical comparison was made by two-sided t-test ($\alpha = 0.05$). $^{**}P < 0.01$: significant difference versus G2, $^{*}P < 0.05$: significant difference versus G1.

weeks in the gel. FA release may be faster from the SC sponge than from the SC film or gel based on the results of *in vitro* release.

Sorbitol, a highly water soluble material, was added to the film as a plasticizer for the *in vivo* study because the SC film without sorbitol easily breaks and was difficult even to cut to a suitable size. The SC film was thus plasticized by the addition of sorbitol resulting in relative less physical strength in PBS depending on the amount of sorbitol (Nishida et al., 2010). Because of this tendency to be weaker under these conditions, films containing sorbitol may be more easily broken than rigid SC films without sorbitol *in vivo*. However, in our study, the drug release rate and biodegradation rate of the film were significantly slower than that of gels without sorbitol. Thus, the addition of sorbitol to the SC film showed little influence on drug release and biodegradation of the SC film and gels.

As for the effect of SC concentration in the gel, FA tended to be sustained longer in the higher concentrated SC gel, compared to the lower concentrated one (Fig. 9, G1 and G2). The release rate of the charged protein drug may therefore be controllable by altering the concentration of SC and/or the dosage form. As a low molecular SC solution that has undergone hydrolyzation is rapidly absorbed upon interperitoneal injection in rats (Takeoka et al., 2004), the degradation rate *in vivo* appears to be affected by the size of SC.

4. Conclusion

The *in vitro* release study of SC film, gel and sponge containing different molecular sizes and concentrations of SC showed that the concentration and dosage form of SC are major factors for effective release of a charged drug protein. Drug release from these three SC preparations was sustained longer when the concentration of SC was greater. Among the three preparations containing a higher concentration of SC, the film was the best *drug releasing* dosage form.

Examination of the biodegradation of SC using an *in vivo* rat model found SC from film and gel encapsulated in collagen-like material from connective tissue, which gradually decreased in size and weight over time. The degradation rate of the SC gel was faster than that of the SC film. Histological observation indicated no marked inflammation upon implantation or injection. The drug remained for more than 6 weeks in the SC film and 3 weeks in the SC gel, and the amount of drug at the implantation site decreased with biodegradation of the SC preparation.

Taken together, the results suggest that SC film, gel and sponge are fairly biocompatible and useful as a *drug releasing* biomaterial for charged protein drugs.

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