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Effect of fibroin sponge coating on in vivo performance of knitted silk small diameter vascular grafts

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ABSTRACT. Vascular grafts under 5 mm or less in diameter are not developed due to a problem caused by early thrombus formation, neointimal hyperplasia, etc. *Bombyx mori* silk fibroin (SF) which has biodegradability and tissue infiltration is focused as tube and coating material of vascular grafts. Coating is an important factor to maintain the strength of the anastomotic region of vascular grafts, and to prevent the blood leak from the vascular grafts after implantation. Therefore, in this research, we focused on the SF concentration of the coating solution, and tissue infiltration and remodeling were compared among each SF concentration. Silk poly (-ethylene) glycol diglycidyl ether (PGDE) coating with concentrations of 1.0%, 2.5%, 5.0%, and 7.5% SF were applied for the double-raschel knitted small-sized vessel with 1.5 mm diameter and 1cm in length. The grafts were implanted in the rat abdominal aorta and removed after 3 weeks or 3 months. Vascular grafts patency was monitored by ultrasound, and morphological evaluation was performed by histopathological examination. SF concentration had no significant effects on the patency rate. However, tissue infiltration was significantly higher in the sample of 2.5% SF in 3 weeks, and 1.0% and 2.5% SF in 3 months. Also, in comparison of length inside of the graft, stenosis were not found in 3 weeks, however, found with 5.0% and 7.5% in 3 months. From these results, it is clear that 2.5% SF coating is the most suitable concentration, based on the characteristics of less stenosis, early tissue infiltration, and less neointimal hyperplasia.

KEYWORDS. artificial vascular graft, endothelial cells, in vivo evaluation, silk fibroin, small diameter

INTRODUCTION

Small diameter artificial vascular grafts play an important role in cardiac and peripheral revascularization. Autologous arterial and venous grafts currently show favorable outcomes for small-diameter bypass grafts.[1] However, autologous arterial and venous grafts

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have material weaknesses, such as vasospasm, limited length, poor quality, and prior use.[2] Therefore, conventional vascular prostheses, such as expanded polytetrafluoroethylene (ePTFE) and polyethylene terephthalate (Dacron), are used for small-diameter bypass grafts. Unfortunately, satisfactory results with long-term patency have not been achieved, especially with grafts with an internal diameter of less than 6 mm.[2-4] This problem appears to be due to the lack of endothelial cells, thrombus formation by foreign bodies, and intimal hyperplasia at the graft-vein anastomotic site due to compliance mismatch with the native vessels.[3,5-8]

Silk fibroin (SF) is a natural polymer that is biocompatible, biodegradable and has various processing abilities, which makes it an ideal material for small diameter artificial vascular grafts.[9-13] Silk fibers are primarily composed of 2 types of proteins, sericin and SF. Sericin is an antigenic gum-like protein surrounding the fibers. SF is the core filaments of silk consisting of highly organized β-sheet crystal- and semi-crystalline regions, which is responsible for silk's elasticity compared with fibers of similar tensile integrity.[12]

In the present study, SF was used to construct artificial vascular grafts. The biological responses to the SF fibers were comparable with the responses to most other commonly used biomaterials. In addition, SF is susceptible to proteolytic degradation *in vivo* and is slowly absorbed.[14-17]

In past research, SF vascular grafts showed one-year patency and the patency rate was higher than that of ePTFE grafts.[18] Furthermore, formation of endothelial cells and smooth muscle cells were found in the early-stages. Although previous studies show that SF is useful for knitted tube material,[19,20] numerous problems still exist. The knitted SF tube cannot maintain its anastomotic strength. In addition, the tube is highly permeable and significant amounts of blood could leak through the graft during/after implantation. To avoid these disadvantages, it is necessary to apply a coating to the tube material.[21] We have used SF as the coating material which has shown its advantages. However, from in our experience the concentration of the SF coating has a great effect on handling specifications during the operation. At high SF concentrations there are difficulties regarding the suture technique. On the other hand, when SF concentrations are too low, the grafts cannot maintain their structural integrity immediately after implantation. The SF concentration affects all of the advantages of SF coating; biocompatibility and improved tissue infiltration. Although it has not been fully evaluated, it seems to have a strong effect on the results of graft implantation.

The purpose of this research was to evaluate the effect of SF concentrations in the coating materials on handling and remodeling of vascular grafts. This was done by conducting physical property tests and implantation studies in the rat abdominal aorta.

RESULTS

Scanning electron microscope images of the 4 types of grafts

Scanning electron microscope (SEM) images of the graft tubes are shown in **Figure 1**.

The inner and outer part of the 4 types of vascular grafts are shown in **Figure 2**. Spaces were observed in the grafts before coating, and decreased as the coating concentrations increased.

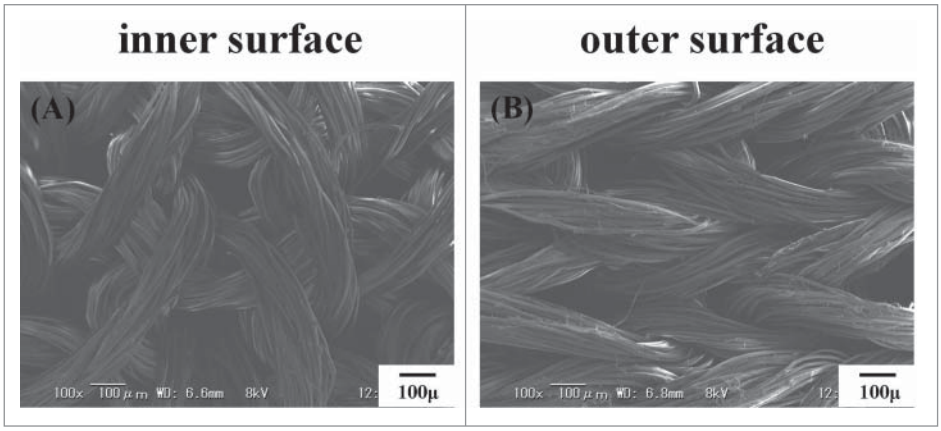
The amount of SF of the 4 types of grafts

The amount of SF in each coating is shown in **Fig. 3A**. The amount of SF was increased as the concentration of SF coating was increased.

In vivo experiments

All 48 implantations were performed successfully with no complications such as uncontrolled bleeding. All rats survived until the scheduled date of explantation and were sacrificed either at 3 weeks or at 3 months. Patency of all implanted grafts was observed by an ultrasound Doppler flow study on day one and just before

FIGURE 1. Scanning electron microscope images of silk fibroin (SF) knitted graft tube. Inner surface (**A**) and outer surface (**B**) are shown.



explantation. Patency rate on Day 1 after implantation was 100%. At three weeks, one 2.5% and one 7.5% SF-coated grafts were occluded. At three months, one graft with 5.0% SF coating was occluded (**Table 1**).

Gross appearance

The abdominal aortic pulse was monitored just before explantation. The patent grafts removed from rats 3 months after implantation had a clean coated layer on the external surface (**Fig. 4**). The gross appearance of the inner lumen of the patent grafts showed no thrombus at 3 weeks or 3 months after implantation.

Physical property test

For *in vitro* graft evaluation, differences in physical properties were observed for all 4 types of vascular grafts. For compressive elastic modulus, 7.5% SF-coating grafts had the highest measurements and significant differences were seen between each of the groups except for between 1.0% and 2.5% SF-coated grafts (**Fig. 3B**). In the longitudinal suture retention test 7.5% SF-coated grafts had the highest scores, and significant differences were confirmed between the 7.5% and other grafts (**Fig. 3C**). Permeability tests showed that the 1.0% SF-coated grafts were most permeable (**Fig. 3D**).

Histopathological examination

The grafts before implantation, graft midportions thickness were checked and HE staining and MTC staining were confirmed (**Fig. 5**).

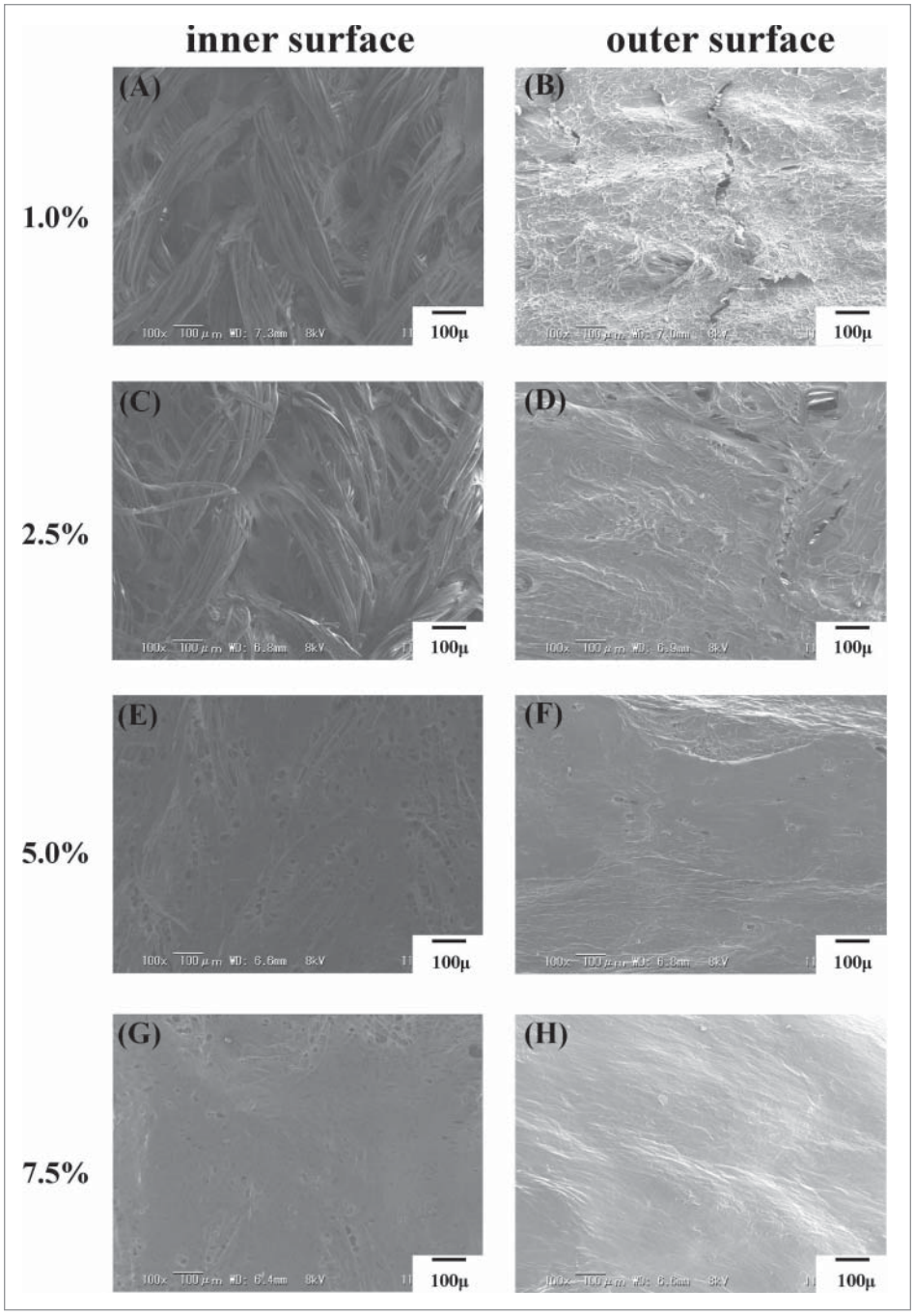
Three weeks after implantation, HE staining confirmed inflammatory cells in all 4 types of vascular grafts (**Fig. 6**). In addition, MTC staining indicated collagen fiber infiltration inside 1.0% and 2.5% SF-coated grafts. On the other hand, collagen fibers were formed around the 5.0% and 7.5% SF-coated grafts.

After three months, most of the coating was degraded in 1.0% SF-coated grafts, and tissue infiltration was confirmed around the SF fibers of the grafts (**Fig. 7**). Collagen fibers decreased in comparison with 2.5% SF-coated grafts after 3 weeks and additional organized infiltration was observed. Furthermore, the collagen fibers around the grafts decreased in the 5.0% and 7.5% SF-coated grafts, with little tissue infiltration inside of these grafts.

Immunostaining with CD31 was used as a marker of endothelial cells and α-smooth muscle actin was used as marker of smooth muscle cells. **Figure 8** shows CD31 and α-smooth muscle actin of 4 types of grafts 3 months after implantation.

Endothelial cells were observed on the almost of the luminal surface except for 7.5%. Smooth muscle cells, which were formed on the media of the blood vessel were formed thinly in 1.0% and 2.5%. On the other hand, a

FIGURE 2. Scanning electron microscope images of coated vascular grafts. Inner and outer surface of the each coating concentration was shown respectively.

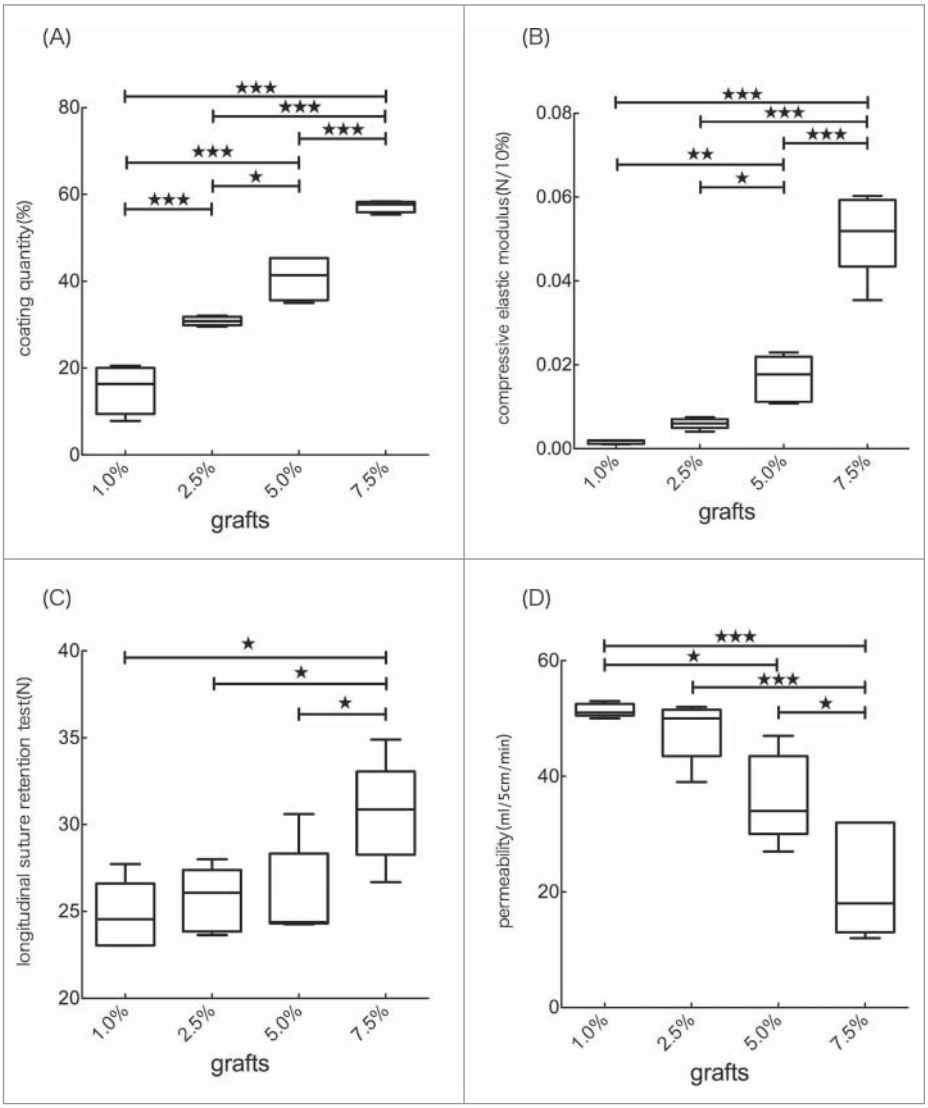


thick layer of smooth muscle cells was formed thick in 5.0% and 7.5%. Because the excessive outbreak of the smooth muscle cells caused neointimal hyperplasia, it was thought to be the cause of stenosis of the grafts.

Imaging analysis

Three weeks after implantation, the 2.5% SF-coated grafts had significantly higher tissue infiltration rates compared to the others (**Fig. 9A**),

FIGURE 3. (**A**) Coating quantity of 4 types of grafts were shown in %. There was significant difference in each grafts. (**B**) Compression test of 4 types of grafts. The values was interpreted as the compressive elastic modulus (N/10%). There was no significant difference between 1.0% and 2.5%. Compressive elastic modulus in 5.0% and 7.5% was significant higher than that in 1.0% and 2.5%. (**C**) Longitudinal suture retention test of 4 types of grafts. The values was interpreted as the retention of strength at anastomotic site. There was significant difference between 7.5% and other grafts. (**D**) Permeability of 4 types of grafts. There was significant difference between 7.5% and other graft.



and 1.0% and 2.5% SF-coated grafts showed significantly higher tissue infiltration compared to 5.0% and 7.5% SF-coated grafts (**Fig. 9B**). There were no significant differences in internal diameter of the grafts at 3 weeks (**Fig. 9C**), however, after 3 months, 5.0% and 7.5% SF-coated grafts showed significantly decreased internal diameter compared to the others (**Fig. 9D**). A single-layer of tissue including smooth muscle cells was found inside the grafts for all grafts types, but it was thin

Table 1. Patency of each graft at 3 weeks and 3 months after implantation

	1.0%	2.5%	5.0%	7.5%
3 weeks	6/6	5/6	6/6	5/6
3 months	6/6	6/6	5/6	6/6

in 1.0% and 2.5% and thick in 5.0% and 7.5% SF-coated grafts.

DISCUSSION

Regardless of their size, implanted vascular grafts are subject to suffer from both thrombus formation in early stages of implantation, and neointimal hyperplasia formation in middle or long-term implantation. They are serious problems especially for small diameter artificial vascular grafts.[22-26]

To improve graft patency, thrombus formation and neointimal hyperplasia should be controlled. Early stage endothelialisation offers a potential solution. In a previous study, it was shown that tissue infiltration in large diameter vascular grafts facilitates endothelialisation.[27] SF is known to have a histotropic character and stimulates cell migration.[18,28] This character could contribute to endothelialisation in the early stages following implantation.

In this study, tissue infiltration in 1.0% and 2.5% SF-coated grafts was significantly higher than in 5.0% and 7.5% SF-coated grafts 3 months after implantation. Most of the coating material had disappeared after 3 months in 1.0% and 2.5% SF-coated grafts. Therefore, we hypothesized that the coating decomposed as tissue infiltration progressed in the earlier stages and coating materials were completely replaced by the host tissue in 3 months.

Surprisingly, tissue infiltration in 2.5% SF-coated grafts was higher than that of 1% SF-coated grafts 3 weeks after implantation. Generally, 1.0% SF coating is more susceptible to decomposition and induces more tissue infiltration compared to 2.5%, however, 2.5% SF coating showed more tissue infiltration 3 weeks after implantation. Histopathological examinations indicated that the decomposition rates of 1.0% and 2.5% SF coatings were similar and that the characteristic of SF to accelerate the migration of tissue likely induces higher tissue infiltration.[18,28] So far, coating materials are considered a negative factor because they act as a physical barrier. However, this study proved that using SF as a coating material could improve tissue infiltration

FIGURE 4. The grafts 3 months after implant just before removed from rat abdominal aortic (**A, C, E, G**). It was able to confirm much tissue around grafts so that coating density was low. Grafts lumen at the mid-portion were checked after removed from rat (**B, D, F, H**). There were many coating around grafts in high density. Also lumen became thick than low density grafts because of neointimal hyperplasia (**F, H**). On the other hand in low density grafts, there were no neointimal hyperplasia and thin inner layer were confirmed and most of coating were decomposed (**B, D**).

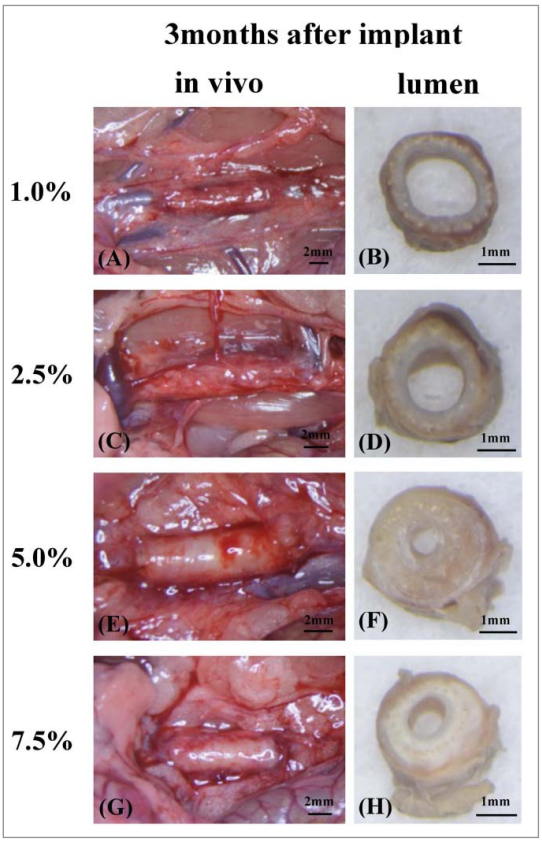
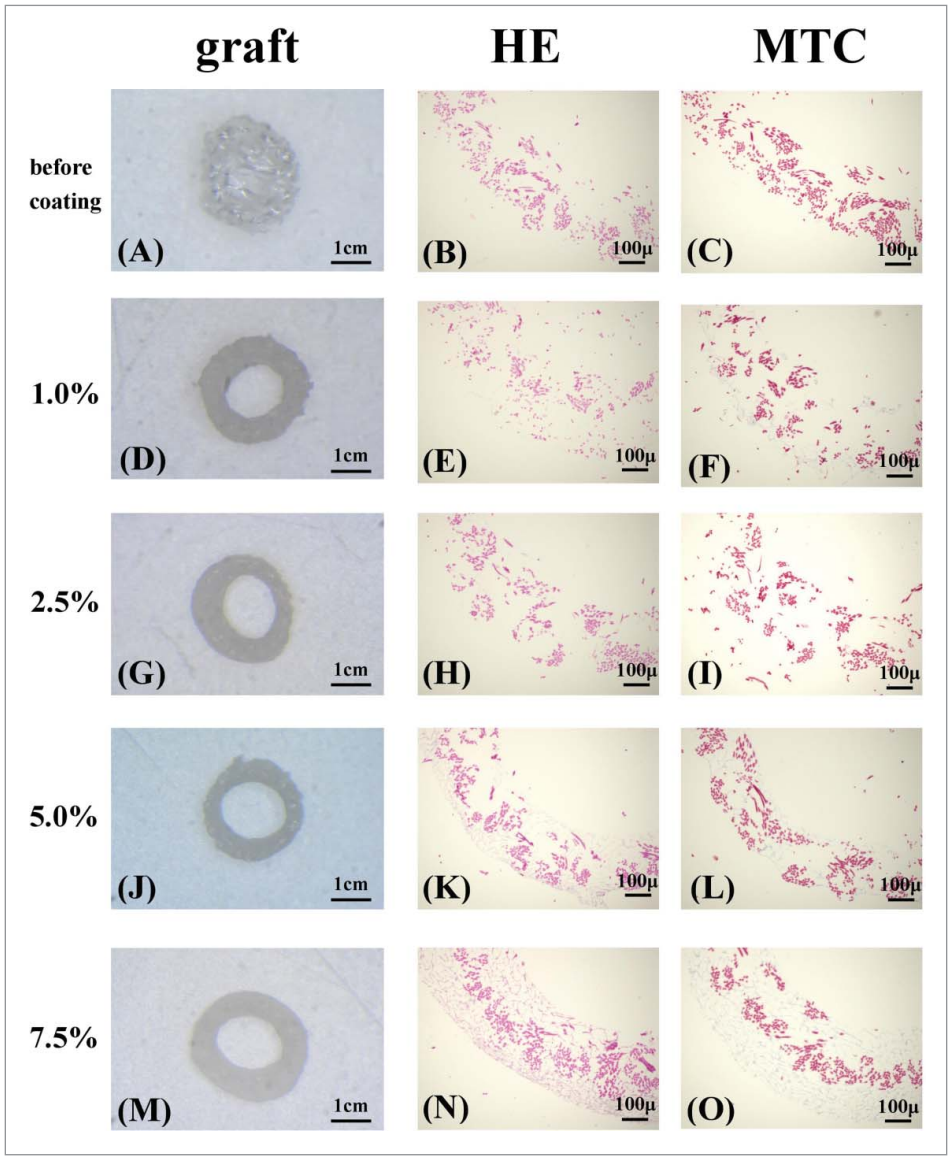


FIGURE 5. The grafts before implant [(A),(D),(G),(J),(M)]. The graft before coating has a lot of space (**A**). The space was covered by each concentration coating. Also there was no difference in the graft thickness (**D, G, J, M**). Histological micrographs of the grafts: Hematoxylin and eosin (HE) staining [before coating (**B**) 1.0% (**E**), 2.5% (**H**), 5.0% (**K**), 7.5% (**N**)], Masson trichrome (MTC) staining [before coating (**C**), 1.0% (**F**), 2.5% (**I**), 5.0% (**L**), 7.5% (**O**)] of grafts before implant.



and endothelialisation if used appropriately. Although SF effectively stimulates cell migration, excessive amounts can disturb tissue infiltration. This study shows that 2.5% SF coating is the optimal concentration to accelerate tissue infiltration.

Neointimal hyperplasia is also a serious problem for small diameter artificial vascular grafts, affecting the mid- to long-term results. Even if endothelialisation is complete, neointimal hyperplasia can cause mid- to long-term stenosis or occlusion. Neointimal hyperplasia is caused by the difference in compliance between native vessels and artificial grafts.[3,5-8] Decomposition of SF coating, tissue infiltration, and fragmentation of the SF fibers were

FIGURE 6. Histological micrographs of the removed grafts: Hematoxylin and eosin (HE) staining [1.0% (**A**), 2.5% (**C**), 5.0% (**E**), 7.5% (**G**)], Masson trichrome (MTC) staining [1.0% (**B**), 2.5% (**D**), 5.0% (**F**), 7.5% (**H**)] of grafts removed 3 weeks after implant.

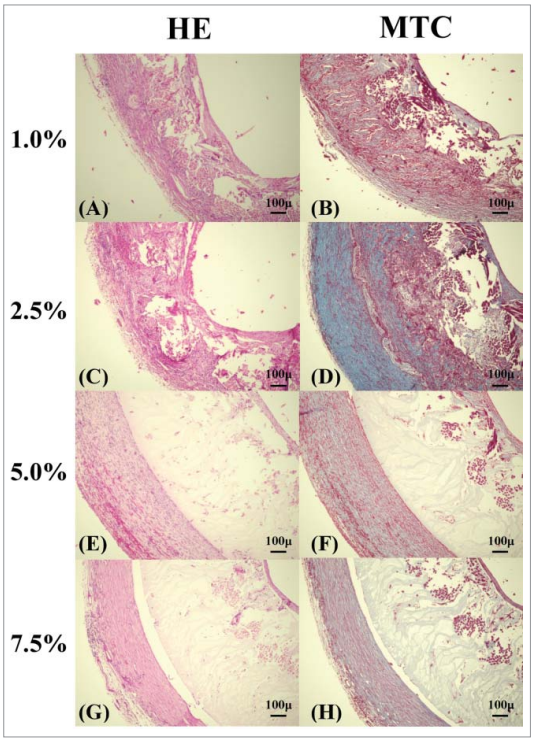
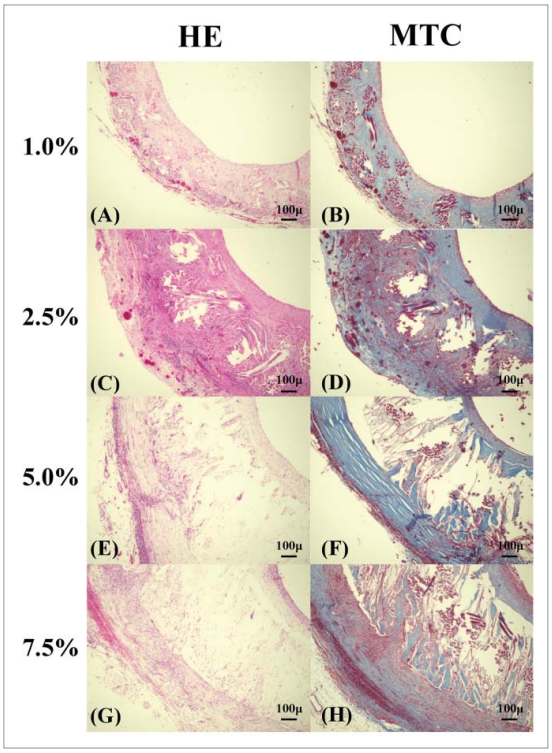


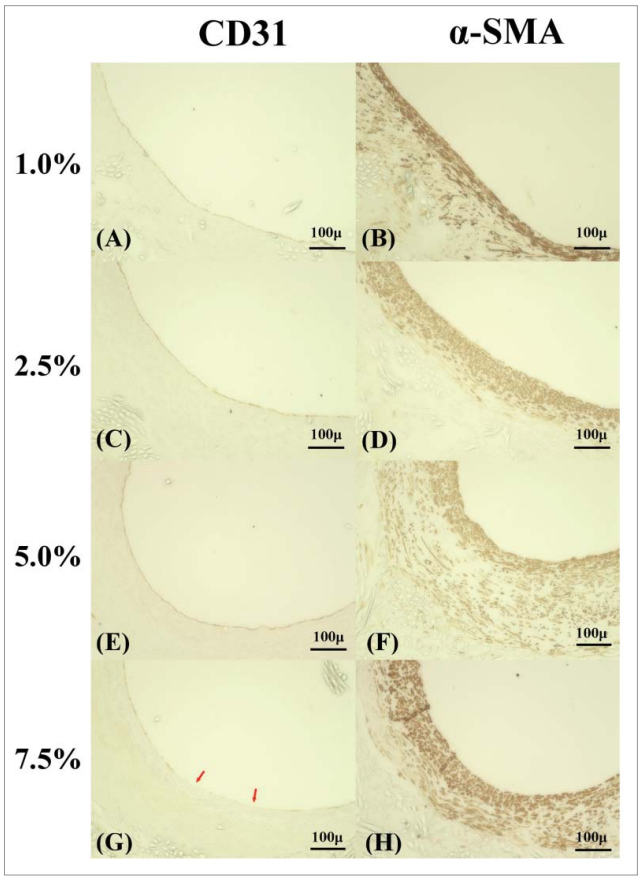
FIGURE 7. Histological micrographs of the removed grafts: Hematoxylin and eosin (HE) staining [1.0% (**A**), 2.5% (**C**), 5.0% (**E**), 7.5% (**G**)], Masson trichrome (MTC) staining [1.0% (**B**), 2.5% (**D**), 5.0% (**F**), 7.5% (**H**)] of grafts removed 3months after implant.



important factors for normalizing the compliance of implanted grafts. In particular, decomposition of SF coating and tissue infiltration are greatly influenced by coating materials. In this study, excessive vascular intima by smooth muscle cells was observed in 5.0% and 7.5% SF coating and was responsible for stenosis after 3 months, whereas no neointimal hyperplasia was observed in 1.0% and 2.5% SF coating. As the grafts we used in this research are 1cm in length, stenosis observed in 5.0% and 7.5% are due to neo-intimal hyperplasia. Overexpression of smooth muscle cells can cause neo-intimal hyperplasia.[5,6,29] Lack of tissue infiltration in 5.0% and 7.5% caused the compliance mismatch. Compliance mismatch can cause and responsible for the turbulent blood flow and also cause overexpression of smooth muscle cells.

After implant the graft endothelial cells is important to prevent thrombus. In this study 3 months after implantation the inner surface was covered withe endothelial cells exclusive of 7.5% grafts. The past study revealed that the tissue infiltration into the grafts will encourage the settlement of endothelial cells.[30] Additionally, the stretched capillary vessels from the tissue around the implanted grafts are thought to have effect to the endothelialization.[31] In the present research, we could not reveal the cause for the lack of endothelial cells in some graft in 7.5%, however tissue infiltration of 7.5% was significantly low compared to that of 1.0% and 2.5%. Also, 3 month after the implantation, in 7.5%, there are inessential tissue proliferation around the graft, which were thought to inhibit the tissue infiltration like a barrier.

FIGURE 8. Endothelial cells and smooth muscle cells in 4 types of grafts 3 months after implant. Endothelial cells were stained by CD31 antibody (**A, C, E, G**), smooth muscle cells were stained by using α-SMA antibody (**B, D, F, H**). (**A, C, E**) Endothelial cells complete coverage the luminal surface in 1.0%, 2.5% and 5.0%. (**G**) Some part of luminal surface in 7.5% wasn't covered by endothelial cells. The red arrow indicates the lack of endothelial cells. (**B, D**) Thinner smooth muscle cells were formed in 1.0% and 2.5%. (**F, H**) Thick smooth muscular layer was formed, and a lumen became stenosis.

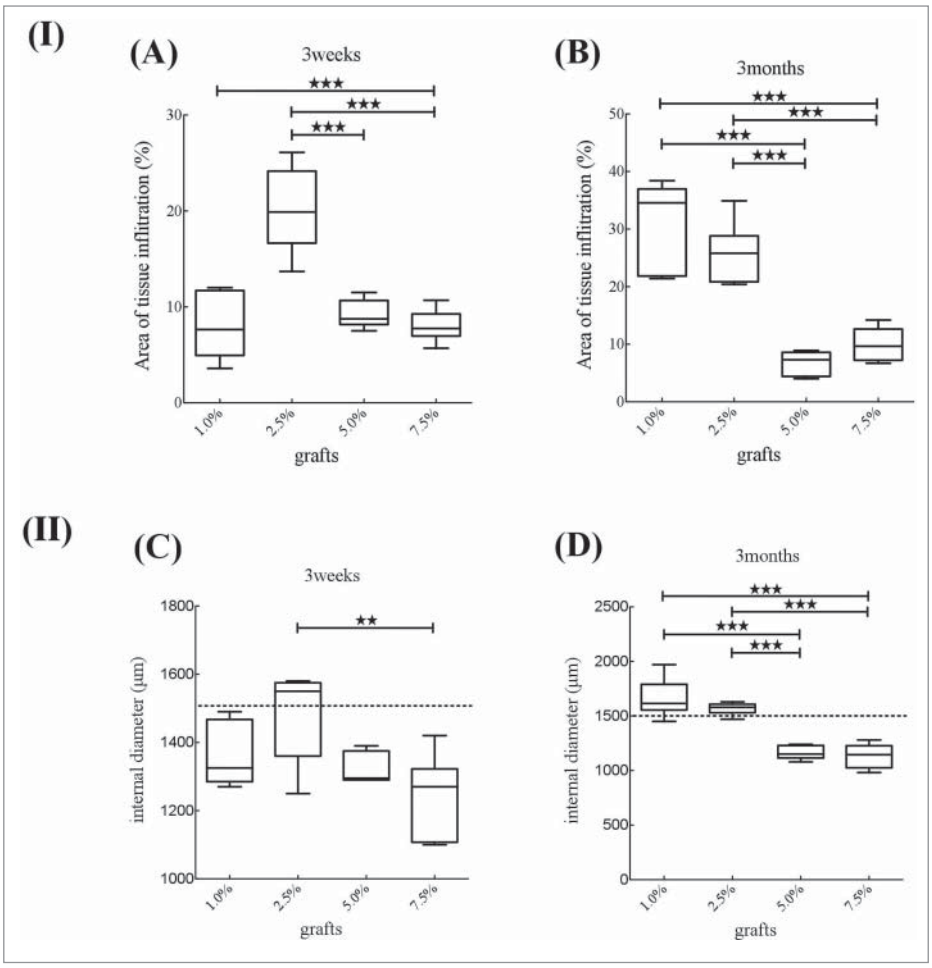


The physical properties of the coating materials are also crucial. The inside diameter of grafts with 1.0% SF coating increased after implantation. Physical property observations further showed that the circumferential tensile strength test and compressive elastic moduli were low in 1.0% SF coating. This indicates that SF tubes cannot retain their form without tissue infiltration and appropriate concentrations of SF. The retention of the suture strength at anastomotic sites also showed the contribution of the SF coating. This strength increased as the coating concentration increased. However, 2.5% SF coating offered sufficient practical strength and a higher concentration was unnecessary.

CONCLUSIONS

To fulfill the demand of small diameter artificial vascular grafts, better tissue infiltration, an unexpandable nature and anastomotic strength are essential factors which can be seriously impacted by the characteristics of coating materials. To optimise the coating material, the composition and concentrations have to be considered.

FIGURE 9. The results of analysis of histopathological examination of the 4 types of grafts. Area of tissue infiltration were calculated in %. (**A**) Three weeks after implantation, graft with 2.5% SF coating shows significantly higher tissue infiltration rate than other grafts. (**B**) Three months after implantation, graft with 1.0% and 2.5% SF coating shows significantly higher tissue infiltration compared to that with 5.0% and 7.5% SF coating. Internal diameter were shown in millimeter. (**C**) Three weeks after implantation, there was significant difference between 2.5% and 7.5%. (**D**) Three months after implantation, 1.0% and 2.5% had significantly longer compared to 5.0% and 7.5%.



MATERIALS AND METHODS

Vascular graft tube

The tubes with 1.5 mm inner diameters were made of Bombyx mori SF fiber, using a computer-controlled double-raschel knitting machine (Fukui Tateami Kogyo Co., Ltd., Japan).[12,32] Double-raschel knitted tubes were shown to have strength and elasticity.[19,33] Scanning electron microscopy (SEM; VE-7800 Keyence, Japan) was used to check the morphology of the SF grafts.[19]

Coating process applied to graft tubes

The SF tube was coated with SF sponge, which was prepared from an aqueous solution of SF and poly (-ethylene) glycol diglycidyl ether (PGDE) as porogen.[19,21,33]

PGDE coating has several material characteristics; it prevents leakage of blood from the graft in the implantation stage, adds softness to the graft, holds tissue, and prevents water infiltrations. Furthermore, after implantation PGDE decomposes rapidly.[34]

The poly (vinylchloride) rod was inserted into the double raschel knitted SF tube and soaked in 1.0%, 2.5%, 5% and 7.5% SF solutions(concentrations that of the SF alone) containing PGDE (SF:PGDE ratio of 1 : 1) for approximately 30 min. The SF graft was then placed under 0.01 MPa reduced pressure until no bubbles were visible from the tube. The tube containing the surrounding the SF/PGDE solution was frozen at -20°C overnight and immersed in distilled water for 3 d to remove the PGDE. The SF graft was sterilized in an autoclave at 120°C for 20 min and kept it in distilled water until the animal implantation experiment.

Weight measurements of coating materials

The weight of coating materials attached to each vascular graft was measured as described by Fuhua et al.[35] First, the weight of the graft tube (cut into 10 mm sections) was measured and recorded as the weight before coating. After coating, graft tubes were allowed to air-dry under ambient conditions, and the weight of each graft tube was measured again. This weight was considered as the weight after coating, and the increase in weight after coating [(the weight of the graft tube after coating - the weight before coating)/ the weight of the graft tube after coating] was calculated.

Animals

Female Sprague-Dawley (SD) rats (Charles River Laboratories, Japan) weighing 200–300 g were used for the *in vivo* study. All rats were kept in micro-isolator cages with a 12-h light/dark cycle. All experimental procedures and protocols were approved by Tokyo University of Agriculture and Technology (Approval number: 26–76), and rats were managed and cared for in accordance with the standards established by Tokyo University of Agriculture and Technology (TUAT) and described in its "Guide for the Care and Use of Laboratory Animals."

Surgical procedure

Four types of vascular grafts (10 mm in length × 1.5 mm inner diameter) were implanted in the rat abdominal aorta. Each type of graft was implanted in 12 rats respectively, 6 of which were removed at 3 weeks and the remaining 6, 3 months after implantation. Thus, 48 rats were used. For graft implantation, rats were anesthetised by intraperitoneal injection of pentobarbital (50 mg/kg body weight). The abdominal aorta was exposed carefully and the aortic branches in this segment were ligated. After intravenous injection of heparin (100 IU/kg), the proximal and distal portions of the infrarenal aorta were clamped with non-crushing vascular clamps. A 10 mm segment of aorta was removed and replaced with a vascular graft by end-to-end anastomosis using interrupted 9–0 monofilament nylon sutures (BEAR, Japan), starting with 2 stay sutures at 180° to each other, then suturing the front wall, followed by the back wall. Each anastomosis required 8–10 stitches. The distal, then the proximal vascular clamps were slowly removed, and flow was restored through the grafts. Total clamp time was 45–60 min. Graft patency was confirmed visually. No anticoagulant or antiplatelet agent was administered postoperatively. The graft patency was monitored by color Doppler imaging (e-flow imaging), and pulse waves were recorded with a 7.5-MHz linear probe and echo-imaging apparatus (Prosound α-10, Hitachi-Aloka, Japan) at one week and again just before explantation, under anesthesia with pentobarbital. Graft diameter, together with any sign of thrombosis or aneurysm formation, were carefully checked by B-mode imaging, and blood flow velocity was measured using a pulsed Doppler flow meter. Before euthanasia, rats underwent a general physical examination to evaluate their

condition. At death, the rats were perfused with 0.9% saline solution through the left ventricle. The grafts were carefully removed together with the surrounding tissue.

Physical property observations

A longitudinal suture retention test was performed to evaluate handling, and a compression test was performed to evaluate usefulness as previously described [21, 25].

When suturing vascular grafts, fraying of the grafts is a major problem. We measured the retention strength of the anastomotic part using EZ graph (Shimadzu, Japan) and evaluated whether it is suitable for operation. We cut the grafts into 20 mm sections and passed the sutures 2 mm from the end, pulled by the clamp 3 mm/min by a 100 N operator cell until break point and analysis.

It is important for graft compliance to be able to adjust to the operability and pulsatile environments after transplantation. Therefore, the universal testing machine, EZ graph (Shimadzu, Japan) was used with specific evaluation software to evaluate circumferential compressive moduli of specimens.[34]

The circumferential compressive elastic modulus of the silk fibroin graft tube was also tested. The test machine which is the same as that used for tensile strength was used. Ring-shaped specimens with an axial length of 10 mm were also prepared for each tube and the outer diameters were measured. Specimens were hydrated in a saline solution for 1 h before testing. The load cell was 5 N and the rate of compression was 2 mm/min. The compressive strength was measured when the specimen was compressed by 10% of the value of the inner diameter. For each test, a stress–strain curve was drawn using the image analysis software installed on the computer as previously described.

The water reservoir was set to apply a hydrostatic pressure to the graft of approximately 120 mmHg. The water permeating through the graft wall was collected and calculated in ml/min/cm^2. Ten specimens were tested for each double-raschel tube.

Histopathological examinations

The grafts were cut transversely into 3 equal pieces and graft mid-portions (~4 mm from the end of the graft) were fixed in 2% glutaraldehyde for histological analyses. Fixed samples (6 μm in thickness) were embedded in paraffin and processed for hematoxylin and eosin (HE) staining and Masson's trichrome (MTC) staining. The sections for immunohistochemical staining were incubated with primary antibodies, including α-smooth muscle actin (clone 1A4; Nichirei Biosciences Inc., Japan) or CD31 mouse anti-rat monoclonal antibody (Lifespan biosciences, United States of America). This was followed by incubation with biotinylated anti-mouse immunoglobulin G secondary antibody (Nichirei Biosciences Inc.), and subsequent color development was done using diaminobenzidine solution (Nichirei Biosciences Inc.). Nuclei were counterstained with hematoxylin.

Imaging analysis

Biological reactions of the stained specimens were visualised with the All-in-One fluorescence microscope (Keyence BZ-9000, Keyence): surrounding fibrosis, tissue infiltration into the graft wall, and the length inside of the graft were examined.

The area of the vascular graft and the area of collagenous fibers inside the graft were also measured, and the percentage of tissue infiltration (area of collagenous fibers inside the graft/area of the vascular graft) was calculated and considered as the degree of biocompatibility (analysis of tissue infiltration).[22,35,36]

The inside length of the graft was divided by π to measure the diameters of the 4 graft types at 3 weeks and 3 months after implantation. This value was regarded as the degree of intimal hyperplasia.[37,38]

Statistical analysis

Data are presented as mean $\pm$ standard error (SD). Comparison of means was performed by one-way analysis of variance, followed by the

Bonferroni post-hoc test. Data analyses were performed using the commercial statistics software package GraphPad Prism (Version 5.0a, GraphPad, San Diego, CA, USA). Statistical significance was defined as $P < 0.05$.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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