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Controlled Release from Multilayer Silk Biomaterial Coatings to Modulate Vascular Cell Responses

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Abstract

A multilayered silk fibroin protein coating system was employed as a drug carrier and delivery system to evaluate vascular cell responses to heparin, paclitaxel, and clopidogrel. The results demonstrated that the silk coating system was an effective system for drug-eluting coatings, such as for stent applications, based on its useful micromechanical properties and biological outcomes. Cell attachment and viability studies with human aortic endothelial cells (HAECs) and human coronary artery smooth muscle cells (HCASMCs) on the drug-incorporated silk coatings demonstrated that paclitaxel and clopidogrel inhibited smooth muscle cell proliferation and retarded endothelial cell proliferation. Heparin loaded silk multilayers promoted HAEC proliferation while inhibiting HCASMC proliferation, desired outcomes for the prevention of restenosis. The preservation of the phenotype of endothelial cells on silk and heparin loaded silk coatings was confirmed with the presence of endothelial markers CD-31, CD-146, vWF and VE-Cadherin using immunocytochemistry assays. A preliminary in-vivo study in a porcine aorta showed integrity of the silk coatings after implantation and the reduction of platelet adhesion on the heparin-loaded silk coatings.

Keywords

silk; fibroin; drug-eluting; stents; coatings; biomaterials; heparin; paclitaxel; clopidogrel

1. Introduction

Stent implantation has improved outcomes following percutaneous transluminal cardiovascular angioplasty (PTCA) used for the treatment of occlusive blood vessel diseases. However, stent implantation is also associated with an excessive proliferation of vascular smooth muscle cells (SMCs), extracellular matrix synthesis, thrombosis, and a chronic inflammatory reaction.^{1–3} These undesirable responses are believed to be initiated by the deep

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vascular injury and the endothelial cell damage generated during the surgical intervention.^{3, 4} The responses are further exacerbated by long term exposure to the foreign metallic device.^{5–7} This complication, known clinically as in-stent restenosis, ultimately results in re-stenosis of the targeted artery.

To alleviate thrombosis and restenosis, one promising approach is to coat the stent with a polymeric layer loaded with therapeutic agents, such as sirolimus, paclitaxel or heparin, which are released gradually at the implantation site to control the biological responses.^{8,9} However, previous studies using stents coated with an array of biodegradable polymers, such as polyglycolic acid (PGA)/polylactic acid (PLA) copolymers, polycaprolactone polyhydroxy (butyrate valerate), polycaprolactone, and poly(ethylene oxide)/polybutylene terephthalate, as well as nonbiodegradable polymers, such as polyurethane, silicone, and poly(ethylene terephthalate), demonstrate increased inflammatory and neointimal responses after implantation in porcine coronary arteries.^{10,11} Recently, natural polymers such as collagen/gelatin, hyaluronan and chitosan were studied as polymer carriers for stent coatings, offering good biocompatibility.^{12–14} However, these polymers present challenges due to their poor mechanical properties and limited ability to sustain stent expansion and deployment, often requiring crosslinking and related treatments to enhance durability, which in turn alters biological responses.^{12–14}

Silks represent a new family of advanced biomaterials, which should help to overcome some of these key limitations in both coated-stent technology and the favorable clinical outcomes possible. A naturally-occurring biopolymer, silk fibroin has high strength, mechanical toughness, robust flexibility, while sustaining excellent biocompatibility, therein presenting considerable utility for a number of difficult-to-solve human therapeutic interventions. Comprehensive studies, both in vitro and in vivo, have demonstrated that silk fibroin is more biocompatible than other commonly used polymeric degradable biomaterials, such as PLA, PGA and collagen.^{15,16} Another important attribute of silks is their processability into different material formats, such as films, gels, nanofibrous membranes and 3-dimensional porous scaffolds, with control of crystalline state (β -sheet content) and morphology to modulate the rate and extent of degradation.^{17–20} The excellent biocompatibility and the ability to control the structural and morphological features of silk proteins in an all-aqueous process render this family of proteins important candidates for biomaterial controlled release applications.

Recently, we demonstrated controlled release nanoscale silk coatings prepared through the stepwise deposition of silk fibroin with small and large molecule model compounds.²¹ The control of the silk fibroin multilayer structure and morphology was successfully used to regulate the release kinetics of the incorporated compounds. The efficacy of silk fibroin thick films as delivery vehicles for bioactive compounds has also been studied using horseradish peroxidase (HRP) and lysozyme (Lys) as model protein drugs.²² The controllable level of crystallinity of silk fibroin provides a basis for this polymer for drug delivery. Crosslinked silk fibroin was also used to achieve sustained release of theophylline, with zero-order kinetics.²³

In the present study, therapeutic compounds currently used to treat restenosis and thrombosis were incorporated into silk coatings with the goal of controlling cell responses. The aim was to reduce smooth muscle cell proliferation, promote endothelial cell growth and minimize platelet adhesion, all relevant to stent functions and required for proper vascular repair following angioplasty. A useful stent would likely entail coating with cell-specific agents that accelerate endothelial regeneration while inhibiting smooth muscle cell hyperplasia and platelet aggregation, thus resulting in a non-thrombogenic stent with early re-endothelialization but without neointimal hyperplasia. Heparin, paclitaxel, and clopidogrel were selected as the pharmacologic components for this study to evaluate their antiproliferative effects on smooth

muscle cells and endothelial cells and their anticoagulation properties in this coating system. Silk fibroin was used as the biomaterial drug carrier for stent coatings to modulate drug release kinetics. Human aortic endothelial cells (HAECs) and human coronary artery smooth muscle cells (HCASMCs) were used to evaluate the cellular responses to the drug-incorporated silk coatings *in-vitro*. The initial cell attachment, cell morphology, growth, and phenotype were investigated. The hemocompatibility of the coatings was examined by human platelet rich plasma assays. A preliminary *in vivo* study was also conducted using a porcine model to evaluate the integrity of the drug-loaded silk coatings after implantation and platelet adhesion.

2. Experimental

2.1. Materials

Cocoons of *B. mori* silkworm silk were kindly supplied by Tajima Shoji Co, (Yokohama, Japan). Heparin sodium salt from porcine intestinal mucosa ($M_w \approx 15,000$ Da) was purchased from Glycomed (Alameda, CA). Paclitaxel, clopidogrel and all other chemicals were of analytical or pharmaceutical grade and purchased from Sigma and Aldrich (St. Louis, MO) and used without further purification. Fresh human platelet rich plasma was purchased from Research Blood Components, L.L.C. (Brighton, MA). Freshly cleaved mica for atomic force microscope (AFM) measurements was from Ted Pella, Inc. (Redding, CA). Quartz microscope slides for nanoindentation test from Quartz Scientific, Inc. (Fairport Harbor, OH). Deionized water (18 M Ω cm) was used in all washing steps and to prepare silk fibroin. Express™ biliary LD stents with a diameter of 6.0 mm were used for *in-vivo* study (Boston Scientific, Natick, MA).

Silk fibroin aqueous stock solutions were prepared as previous described.²⁴ Briefly, cocoons of *B. mori* were boiled for 20 min in an aqueous solution of 0.02 M Na₂CO₃, and then rinsed thoroughly with distilled water to extract the glue-like sericin proteins. The extracted silk fibroin was then dissolved in 9.3 M LiBr solution at 60°C for 4 h, yielding a 20% (w/v) solution. This solution was dialyzed against distilled water using a Slide-a-Lyzer dialysis cassette (MWCO 3,500, Pierce) at room temperature for 48 h to remove the salt. The dialysate was centrifuged 2 times, each at -20°C for 20 min, to remove impurities and the aggregates that formed during dialysis. The final concentration of silk fibroin aqueous solution was approximately 8% (wt/v). This concentration was determined by weighing the residual solid of a known volume of solution after drying at 60°C for 24 h.

Silk solutions used for coating formation were prepared by diluting the stock silk solution with deionized (DI) water to a concentration of 2 mg/ml. Mixed heparin and silk solutions were prepared by dissolving heparin in DI water first and then adding this to the silk solution to obtain a final concentrations of heparin of 1.0 and 25 mg/ml for low dose and high dose loading, respectively, in which the silk concentration was 2 mg/ml. Paclitaxel was dissolved in ethanol at concentration of 0.50 mg/ml and 3.0 mg/ml for low dose and high dose loading, respectively. Clopidogrel solution was prepared with methanol at a concentration of 20 mg/ml. The selection of the concentration was based on literature values^{25,26} for preliminary screening.

2.2. Preparation of multilayer silk coatings

The multilayer silk coatings were performed on 24-well tissue culture plates for cell culture study and metallic stents for the *in-vivo* study. The targeted multilayer structures of the designed drug-incorporated coatings were as follows: six layers of silk [(silk)₆], six layers of heparin +silk [(HP+silk)₆], six bilayers of heparin+silk/clopidogrel [(HP+silk)/CP]₆, and six bilayers of silk/paclitaxel [(silk/PTX)₆], where the subscript corresponds to the number of layer or bilayers therein. Each group of drug-loaded multilayer silk coatings consisted of both low dose and high dose. The thicknesses of these coatings were in the range of 30 to 70 nm measured

by AFM technique as described in a previous study.²¹ A 6-layered modality was chosen in accordance to the previous release study using model compounds.²¹

Simplified notes for the above samples were as follows: Silk (six layers of silk), HPL (six layers of heparin+silk low dose), HPL+CP (six bilayers of heparin+silk/clopidogrel low dose), HPH (six layers of heparin+silk high dose), HPH+CP (six bilayers of heparin+silk/clopidogrel high dose), PTXL (six bilayers of silk/paclitaxel low dose), and PTXH (six bilayers of silk/paclitaxel high dose). A summary of the samples used in this study is list in Table 1.

For tissue culture plate surface coating, the buildup of the multilayer silk and multilayer heparin/silk were accomplished by the repeated addition of the respective solution onto the tissue culture plate well, using a 5 min exposure time. Between each step, the solution was removed and the culture plate well was rinsed with deionized water for 1 min and dried with a gentle flow of nitrogen gas. The incorporation of water-insoluble drugs, clopidogrel and paclitaxel, was carried out by adding 10 μ l of the respective drug solution between silk layers and drying with a gentle flow of nitrogen gas. The coatings of the metallic stents were performed by dipping the stents in the heparin/silk solution and clopidogrel solution, alternately, for 5 min each. Between each step, the stents were rinsed with deionized water for 1 min and dried with a gentle flow of nitrogen gas. The number of layers or bilayers was six for all the samples in this study.

2.3. Surface morphology and micromechanical properties of the multilayer coatings

The surface morphology of the drug-loaded silk coatings before and after incubation in PBS for 24 h were characterized by Atomic Force Microscopy (AFM) using a Digital Instrument Dimension 3100 system from Veeco (Woodbury, NY) in tapping mode.

The coating adhesion and wear resistance were estimated by microscratch measurements using a Vickers indenter and AFM. Both thin (< 100 nm) and thick (~ 5 μ m) silk coating samples were studied. In the microscratch measurement, a Vickers indenter (inverse pyramid, 136° face to face angle) was used with a face-on orientation. The indenter (with an edge first) was run across the coating surface with a linearly increasing load from 0 up to 200 mN and 400 mN at a rate of 10 mN/s and a horizontal displacement rate of 10 μ m/s for a scratch distance of 2 mm. Experiments were carried out using an experimental setup constructed in-house, which allows for the control of the normal load by a piezoelectric actuator concurrent with lateral movement of the sample stage. The scratch trace was analyzed by AFM. The friction coefficient between silk coating and metal surface was obtained from both techniques.²⁷

2.4. In vitro cell seeding and culture

Cell studies were carried out to assess cellular responses to the multilayer drug-incorporated silk coatings using human endothelial cells and smooth muscle cells, the primary cells of the vascular system. The human aortic endothelial cells (HAECs) (Clonetics, Walkersville, ML), passage 5, and immortalized human coronary artery smooth muscle cells (HCASMCs), passage 13 (24 years old donor, female, no vascular disease), (Tufts-New England Medical Center, Boston, MA) were used. The coated 24-well culture plates were sterilized by UV light in a cell culture hood for 20 min. Cell culture media for HAECs and HCASMCs were Endothelial Growth Medium-2 (EGM-2, Cambrex Bio Science, Walkersville, ML) supplemented with 10% FBS, and low glucose Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Carlsbad, CA) with 10% FBS, penicillin/streptomycin and additional L-glutamine, respectively. The cells were seeded at 10^4 cells/cm² in culture media, EGM-2 and DMEM, for HAECs and HCASMCs, respectively, at 37°C in a humidified incubator supplemented with 5% CO₂. One ml of medium was used for each well and the medium was changed every 2~3 days over 16 days for the HAECs and 28 days for the HCASMCs, respectively.

2.5. Cell attachment and proliferation studies

The assessments of the attachment and proliferation associated with antigens of HAECs and HCASMCs were performed using alamarBlue™, which measures metabolic activity, via fluorometric analysis (BioSource International, Inc. Camarillo, CA). High levels of metabolic activity indicate cellular viability and proliferation, and low levels imply cellular toxicity. As with tetrazolium salts, alamarBlue™ monitors the reducing environment of the proliferating cell. The assay utilizes an oxidation-reduction (REDOX) indicator that changes color in response to chemical reduction of the growth medium caused by cell metabolism. At each predetermined time point, the cells in culture were incubated with 10% alamarBlue™ in culture medium at 37°C for 1 h. Metabolism was evaluated by exciting at 540 nm and measuring emission at 590 nm on a spectrofluorimeter.

2.6. Endothelial cell phenotype study

Immunohistochemistry for the endothelial cell markers CD-31, CD-146, vWF, and VE-Cadherin was performed using VECTASTAIN Elite ABC Reagent (Vector Laboratories, Burlingame, CA) following the manufacturers protocol. After 3 days of culture at a seeding density of 6×10^3 cells/cm² on the unmodified tissue culture plates, silk coated plates, and heparin-incorporated silk coated plates, HCAECs were washed with PBS-Tween solution for 5 min, fixed with in ice-cold methanol and acetone mixture (1:1) for 10 min at 4°C and blocked with horse serum for 20 min. HCAECs were then incubated with primary antibodies overnight at 4°C in a hydration chamber. After washing with PBS-Tween 3 times, the cells were incubated with diluted biotinylated secondary antibody solution for 30 min, followed by washing and counterstaining with VECTOR® HEMATOXYLIN QS for 5–45 seconds. The immunostained cell samples were then viewed under an optical microscope. The controls were performed following the same procedure without biotinylated secondary antibody incubation.

2.7. Hematocompatibility

The evaluation of hematocompatibility of the drug-loaded silk coatings was carried out by examining platelet adhesion. Unloaded silk coatings and glass slides were used as positive control substrates. Tissue culture plates were used as negative controls. Briefly, to determine platelet adhesion, the samples were conditioned in PBS buffer for 20 min. The samples were then submerged with freshly prepared platelet rich plasma ($>2.5 \times 10^6$ platelets/ml) and incubated at 37°C for 30 min. After washing gently with buffer 3 times to remove non-adhering platelets, the air-dried samples were stained with undiluted Geishma stain for 1~2 min followed by 3 times PBS washes and were examined by optical microscopy. The average numbers of platelets in five random regions were recorded manually for each sample. In preparation for SEM, the adhered platelets were fixed with formalin (20 min, RT), dehydrated in a graded series of ethanol (20–100% v/v), dried in air, and coated with gold over 60 s in a sputter-coater. The morphology of the platelets was examined using SEM.

2.8. Balloon-expansion test

Stents coated with heparin-loaded silk were mounted on a commercial angioplasty balloon (Boston Scientific, Boston, MA) and the balloons inflated with DI-water to a pressure of about 10 atm for 30 s, deflated and slowly withdrawn. Subsequently, the integrity of the coating on the stent was examined by SEM.

2.9. In vivo study

Yorkshire swine, 27 to 32 kg body weight, were used for the in vivo study according to procedures approved by the Institutional Animal Care and Use Committee, in accordance with the "Guide for the Care and Use of Laboratory Animals" (National Research Council. Guide for the Care and Use of Laboratory Animals. Washington, DC: National Academy Press; 1996.)

Metallic stents, bare and coated with heparin/silk+ clopidogrel, two for each group, were implanted in the abdominal aorta in a porcine model for 2.5 h. The explanted stents and the stented aorta were rinsed with saline buffer and fixed in 2 % formalin solution. The stents were then dehydrated in a graded series of ethanol (20–100 % v/v), dried in air and coated with gold over 60 s in a sputter coater and examined by SEM. For histopathological analysis, the aorta were embedded in Technovit 9100 and cut into 4–6 μm -thick slices. Three sections of drug-loaded stented and bare metallic stented aorta were stained with hematoxylin-eosin and examined under optical microscope for general assessment of the histological appearance to check for abnormal vascular reactivity to the stent interventions.

2.9. Statistical analysis

All assays were performed in triplicate and results reported as means $\pm$ standard deviation. Independent sample t-tests were used to determine statistical significance; the significance for all tests was set at $p < 0.05$ (*) and $p < 0.01$ (**).

3. Results and Discussions

3.1. Micromechanical Properties of the Multilayer Coatings

With stent coating applications, the problem of de-lamination is particularly challenging and requires full consideration of the interface between the coating and underlying substrate. In the nanoindentation scratch test, the linear increase loading cycle applied during the microscratch test typically gives rise to three different regimes.²⁸ The first is the micro-ductile regime, corresponding to 'plastic' deformation under the indenter. Sub-surface lateral cracks may also appear in this first regime. The second regime, occurring when the load increases, is called the micro-cracking regime. This term refers to chip formation as a consequence of the intersection of lateral cracks with the surface. The third regime is termed as the microabrasive regime, and is characterized by the formation of debris. All transitions from one regime to another occur at well defined loads.

To examine the biomechanical properties of the silk-drug multilayers, microscratch measurements using a Vickers indenter and AFM were performed. Figure 1 shows the AFM images of the microabrasive regime of the silk coatings with different loads. In general, the higher the applied normal load, the larger the damaged zone on the sample. Interestingly, this is not the case for the silk coating samples under investigation. The widths of the scratches under 200 mN and 400 mN loads are both ~ 30 μm . The fact that the damaged zone width did not increase when the normal load increased can be explained that the silk film played a role of absorber, causing the damage on the substrate to be limited.

The dynamic friction coefficient of the silk fibroin coatings obtained was 0.17 to 0.20. A comparison of the friction coefficient of silk with other materials is shown in Table 2.^{29,30} Most polymers have coefficients of friction in the range 0.2 to 0.6, with Teflon being as low as 0.04. The lower friction coefficient of silk when compared with most polymer materials is an indication of good adhesion and wear resistance.^{29,30}

3.2. Cell attachment and proliferation study

The effect of the drug-incorporated silk coatings on initial cell attachment was investigated 90 min after cell seeding. Attachment of HAECs and HCASMCs were evaluated by alamarBlue™ cell viability assay on different surfaces as shown in Figure 2. For HAECs, a significant reduction in adhered cells was found when the coatings loaded with clopidogrel and paclitaxel were compared with the TCP control. For HCASMCs, clopidogrel-loaded coatings exhibited significantly reduced cell attachment.

It has been recognized that a functional stent would accelerate endothelial regeneration while inhibiting smooth muscle cell hyperplasia.⁴

The assessment of the response of endothelial and smooth muscle cells to the drug-loaded silk coating *in vitro* can provide the context for more effective system designs *in vivo*.

The viability of HAECs was determined at days 1, 3, 8 and 16 cultured on the eight different surfaces after cell seeding (Figure 3), using alamarBlue to measure metabolic activity as a marker of viability. HAEC viability on the unloaded silk and heparin-loaded silk was not significantly different when compared with the tissue culture plate control from day 3 onwards. However, all of the clopidogrel- and paclitaxel-loaded silk coatings were toxic to the cells, showing low viabilities.

To ascertain the effect of the stent coatings on cell proliferation, we measured cell proliferation on the coatings. In the case of HCASMCs (Figure 4), a significant reduction in cell proliferation was observed for all samples when compared with the TCP control from day 14 onwards. Similarly, all the coatings loaded with clopidogrel and paclitaxel prohibited HCASMC growth. Figure 5 shows optical images of the HAECs on day 10 and HCASMCs on day 28 on TCP, silk, heparin low, and heparin high surfaces. Both type of cells exhibited normal morphology on silk and heparin-loaded silk surfaces comparable to the control TCP surface. Confluent HAECs were observed on the TCP, silk and heparin-loaded silk coatings. However, a significant reduction in HCASMC proliferation was observed on the silk and heparin-loaded silk surfaces. No live cells were observed on the clopidogrel- and paclitaxel- loaded silk coatings for both cell types. This observation correlated with the results from the cell viability assays. The results indicate that heparin loaded silk multilayers promoted endothelial cell and inhibited smooth muscle cell proliferation, desirable outcomes related to control of restenosis. Although paclitaxel inhibits smooth muscle cell proliferation, it also retards endothelial cell regeneration, potentially preventing the restoration of vasculature morphologic and functional integrity. Clopidogrel is an anti-platelet compound that is often used orally along with aspirin for the prevention of thromboembolism after placement of intravascular stents. In the present study our aim was to incorporate Clopidogrel into the coating to achieve more effective drug delivery. To date, the effects of Clopidogrel on the proliferation of endothelial cell and smooth muscle cell has not been fully investigated. It was reported that Clopidogrel inhibited platelet-dependent mitogenesis in vascular smooth muscle cells.³¹ Another study showed that Thienopyridine and Ticlopidine, similar drugs to Clopidogrel, inhibited human saphenous vein endothelial cell proliferation and cell migration during lesion repair in a dose-dependent manner.³² Further studies are needed to investigate the effects of the delivery dose of Clopidogrel and its conjugated outcomes with heparin on endothelial cells and smooth muscle cells both *in vitro* and *in vivo*. At least from the results presented here, it is clear that at the doses used there was toxicity to the cells studied, findings that should be considered in future studies to assess delivery modes with this drug, both *in vitro* and *in vivo*. Strategies to develop multi-agent release systems are desired with the triple functions of promoting endothelial cell regeneration, inhibiting smooth muscle cell proliferation, and minimizing platelet adhesion.

3.3. Endothelial cell phenotype study

To determine if the endothelial cells retain their phenotype after plating onto silk-drug multilayers, surface proteins characteristically expressed by endothelial cells were studied using immunocytochemistry. Endothelial cell adhesion molecules, CD-31, CD-146, vWF, and VE-Cadherin are universal EC markers, the expression of which can represent the differentiation status of endothelial cells. Figure 6 contains micrographs of immunostained HAECs cultured on TCP, silk, heparin/silk low dose, and heparin/silk high dose multilayers. HAECs cultured on all the samples showed positive phenotypes compared with the control.

The results indicated the preservation of EC characteristic phenotype on silk and heparin loaded silk coatings.

3.4. Hematocompatibility

In-stent restenosis is caused by neointimal hyperplasia which may be stimulated by several mitogenic agents including platelet-derived growth factor (PDGF).^{33,34} Animal studies with heparin-coated stents have shown a significant reduction in stent thrombosis when compared to untreated stents,³⁵ and results from human studies have also been promising.³⁶ Therefore, incorporation of heparin in silk for stent coatings might be an attractive approach to improving blood compatibility and reducing the growth of tissue in the stent. In this study, platelet count and morphology were used to evaluate the blood compatibility of the heparin-incorporated silk fibroin coatings. Typical images of surface-platelet rich plasma-contacted samples are shown in Figure 7: (A) glass positive control, (B & G) TCP negative control, (C & H) silk coating without drug, (D) silk coating loaded with low dose heparin, (E) silk coating loaded with high dose heparin. The summary of relative number of platelets on each sample type is shown in F. The adhesion on the heparin-loaded coatings was similar to TCP negative control but was significantly less compared to bare glass and silk coating without drug, an indication of the feasibility of drug loading and release from silk coatings for blood compatible surfaces and specific control (platelet adhesion). SEM images showed discrete and rounded platelet morphology for the attached platelets on the TCP control and silk coated surface. The low platelet spreading indicates that the surface does not activate these cells, reducing the likelihood of thrombus formation upon stent contact with blood.

3.5 Stent expansion and in vivo study

To determine if silk-coated stents maintained their ability to permit endothelial regeneration and hematocompatibility in vivo, SEM images of the silk coatings on Express™ Biliary I.D. metallic stents pre- and post-balloon expansion were prepared (Figure 8). In addition, fluorescent microscope images of the silk coatings with incorporated FITC-labeled dexamethasone for visualization demonstrated the conformal thin coatings on these devices with the aqueous silk solution shown in the top row (Figure 8). The silk coatings were smooth and uniform with no obvious cracking, flaking or delamination. SEM images of explanted stent surfaces in contact with porcine aorta wall for 2.5 hours are shown in the bottom two rows (Figure 8). The coated stents (silk coating loaded with high dose heparin) had significantly reduced platelet adhesion than uncoated stents. Histological analysis of the stented aorta revealed no noticeable differences between the two groups of samples (Figure 9), although this may be due to the very short implantation time. Longer term studies will be required to more fully evaluate the effectiveness of heparin-loaded silk coatings in controlling vascular responses.

4. Conclusions

The feasibility of utilizing drug-loaded silk fibroin for vascular stent coatings was assessed in the present work. The ability of silk coatings to regulate the adhesion, viability and growth of human aortic endothelial cells and human coronary artery smooth muscle cells was demonstrated *in vitro*. A preliminary *in vivo* study using a porcine model supported the *in vitro* results in terms of the integrity of the silk coatings, maintenance of the endothelial cell phenotype, and the reduction of platelet adhesion on the drug-loaded silk coatings. Future investigations will evaluate the long term effects of drug-loaded silk coatings both *in vitro* and *in vivo*.

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Figure 1.
AFM images of the microabrasive regime of the scratch trace with different loads: A = 200 mN; B = 400 mN.

Figure 2.
Human aortic endothelial cell (EC) and human coronary artery smooth muscle cell (SMC) attachment after 90 min: $p < 0.05$ (*); $P < 0.01$ (**) compared with TCP controls.

Figure 3.

Human aortic endothelial cell (HAEC) proliferation using alamarBlue™. No significant difference was observed for the cell responses among TCP, Silk, HPL, and HPH surfaces at the same time points. Coatings loaded with clopidogrel and paclitaxel were toxic to the HAECs.

Figure 4.

Human coronary artery smooth muscle cell (HCASMC) proliferation using alamarBlue™. A significant reduction in cell viability was observed for all the samples when compared with TCP controls ($p < 0.01$).

Figure 5. Photomicrographs of endothelial cells (A, B, C, and D) on day 10 and smooth muscle cells (E, F, G, and H) on day 28, respectively, on the different surfaces: (A, E) TCP; (B, F) Silk; (C, G) HPL; (D, H) HPH. Scale bars = 100 µm.

Figure 6.

Immunocytochemistry staining of HAECs on TCP, Silk, HPL, and HPH coatings. Scale bars = 20 μm. Abbreviations: CD 31: PECAM; vWF: von Willebrand factor; VE-C: vascular endothelial cadherin.

Figure 7.

Platelet adhesion microscope images (A, B, C, D, and E) and SEM images (G and H). (A) glass positive control, (B & G) TCP negative control, (C & H) Silk, (D) HPL, (E) HPH. Summary of data in F. Scale bars: A, B, C, D, and E = 20 μm ; G & H = 10 μm .

Figure 8.

(Top Row) SEM micrographs metallic stents coated with fluorescent dexamethasone-loaded silk fibroin pre- and post-expansion (A&B) and fluorescence microscopic image (C). (Bottom Two Rows) SEM images of the surfaces of the heparin/silk coated stents (D, E, and F) and bare metallic stents (G, H, and I) explanted from a porcine model after 2.5 h implantation in the ascending aorta. Scale bars: A: 1 mm; B: 2 mm; C: 500 μm; D & G: 200 μm; E & H: 100 μm; F & I: 100 μm.

Figure 9.
H&E staining of stented aorta. Top row: bare stent control; bottom row: silk/heparin coated stent. No noticeable difference was observed (implant time was 2.5 h).

Table 1

Drug-incorporated silk coatings.

Simplified note	Multilayer structure	Drug loading density
TCP	Tissue culture plate	0
Silk	(silk) ₆	0
HPL	(heparin+silk) ₆ low dose	1 µg/mm ² heparin
HPL+CP	[(heparin+silk)/clopidogrel] low dose	1 µg/mm ² heparin; 1 µg/mm ² clopidogrel
HPH	(heparin+silk) ₆ high dose	300 µg/mm ² heparin
HPH+CP	[(heparin+silk)/clopidogrel] high dose	300 µg/mm ² heparin; 1 µg/mm ² clopidogrel
PTXL	(silk/paclitaxel) ₆ -silk low dose	0.3 µg/mm ² paclitaxel
PTXH	(silk/paclitaxel) ₆ -silk high dose	2 µg/mm ² paclitaxel

Table 2

Dynamic friction coefficient of interfaces between different materials and steel.

Material	Silk coating	Teflon	Nylon 6,6	Polystyrene
Friction coefficient	0.17 ~ 0.20	0.04	0.20~ 0.50	0.30 ~ 0.35