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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 (SMC) 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; Stent; 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 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,

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

Silk fibroin aqueous stock solutions were prepared as described previously [24]. Briefly, cocoons of *B. mori* were boiled for 20 min in an aqueous solution of 0.02 M Na_2CO_3, 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 3500, Pierce) at room temperature for 48 h to remove the salt. The dialysate was centrifuged two 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 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 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