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Silk biomaterials functionalized with recombinant domain V of human perlecan modulate endothelial cell and platelet interactions for vascular applications

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ABSTRACT

Modulation of endothelial cell and platelet interactions is an essential feature of vascular materials. Silk biomaterials were functionalized with recombinantly expressed domain V of human perlecan, an essential vascular proteoglycan involved in vasculogenesis, angiogenesis and wound healing, using passive adsorption or covalent cross-linking via carbodiimide chemistry. The orientation of domain V on the surface of silk biomaterials was modulated by the immobilization technique and glycosaminoglycan chains played an essential role in the proteoglycan presentation on the material surface. Covalent immobilization supported improved integrin binding site presentation to endothelial cells compared to passive adsorption in the presence of glycosaminoglycan chains, but removal of glycosaminoglycan chains resulted in reduced integrin site availability and thus cell binding. Silk biomaterials covalently functionalized with domain V supported endothelial cell adhesion, spreading and proliferation and were anti-adhesive for platelets, making them promising surfaces for the development of the next-generation vascular grafts.

1. Introduction

Cardiovascular disease is the leading cause of mortality worldwide, with coronary artery disease the largest contributor to this epidemic, causing 7.4 million deaths in 2012 [1]. In the absence of a suitable native vessel, synthetic polymer grafts are materials of choice in surgical vascular bypass surgery. While these grafts are adequate for large vessel replacement, they are characterized by high re-occlusion rates in small diameter vessels [2]. To achieve long-term patency, vascular graft materials should support endothelial cell interactions, while inhibiting platelet adhesion and smooth muscle cell proliferation. Biomimetic approaches have the potential to realize this goal by replicating important extracellular matrix cues on graft material surfaces to modulate cell interactions and graft performance [3]. However, these approaches are constrained by the inherent mechanical mismatch between the native vessel and synthetic vascular grafts [4].

Silk-based biomaterials are emerging as promising platforms for bioengineering and regeneration of many tissues, including blood vessels. Silk is a versatile, cell compatible, biodegradable protein that can be processed into a range of material formats with highly tunable mechanical properties [5,6].

The most commonly utilised silks are derived from domesticated *Bombyx mori* silkworm cocoons due to their abundance and established farming methods. Silk-based vascular grafts have been engineered via electrospinning, gel spinning and film casting and have been evaluated in vitro and in vivo [7–10]. However, *Bombyx mori* silk has no inherent cell-binding domains and much work has gone into functionalizing silk biomaterials to incorporate cell interacting moieties [11].

Perlecan (heparan sulfate proteoglycan 2, HSPG2) is a large, conserved extracellular matrix proteoglycan expressed in basement membranes and in many mesenchymal and connective mammalian tissues. Perlecan plays a crucial role in a range of developmental and biological processes, including vasculogenesis, angiogenesis and wound healing [12,13]. Blocking of perlecan expression in early embryogenesis leads to severe cardiovascular defects in mammals [14–16]. The functional versatility of perlecan stems from its ability to bind a range of biological molecules, including extracellular matrix proteins, growth factors and mitogens and cell receptors [17]. The 470 kDa perlecan protein core consists of five structural domains with four sites for decoration with glycosaminoglycan chains, found on domains I and V. Perlecan plays a major role in angiogenesis through its C-terminal α2β1 integrin binding site and N-terminal heparan sulfate chain-

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mediated growth factor signaling. In particular, perlecan binds two highly potent pro-angiogenic growth factors, fibroblast growth factor 2 and vascular endothelial growth factor, which are known to stimulate endothelial cell survival, proliferation, migration and differentiation [13].

Perlecan supports endothelial cell binding and proliferation in the presence and absence of glycosaminoglycan chains, while smooth muscle cells only bind to perlecan in the absence of glycosaminoglycans [18]. The adhesion of both cell types is mediated via domains III and V [18]. Perlecan was found to be anti-adhesive to platelets in the presence of heparan sulfate chains, but when these glycosaminoglycan chains were removed, perlecan supported platelet adhesion and aggregation [19]. Endothelial cell-derived perlecan presented on the surface of synthetic expanded polytetrafluoroethylene (ePTFE) vascular grafts was shown to accelerate and promote graft endothelialization in a sheep carotid interposition model, highlighting its potential in vascular graft development [19]. However, due to prohibitive yield, cost and quality of perlecan isolated from primary cell and tissue sources, recombinant forms of perlecan are attractive means of producing clinically relevant quantities of purified proteoglycan. While a recombinant form of full-length perlecan has been expressed in HEK-293 cells [20], the large size of this product makes expression of individual perlecan domains more feasible.

Domain V of human perlecan was recombinantly expressed as a proteoglycan decorated with heparan sulfate and chondroitin sulfate glycosaminoglycan chains [21,22]. This C-terminal domain of perlecan is of particular interest in vascular research as contains the endothelial cell $\alpha 2\beta 1$ integrin binding site and the only glycosaminoglycan binding site outside domain I. Recombinant domain V was shown to support endothelial cell binding to the same extent as full-length perlecan, but did not support smooth muscle cell binding [18].

In the current study, silk biomaterials were functionalized with recombinant domain V using passive adsorption and covalent cross-linking in order to assess its utility in modulating endothelial cell and platelet interactions on the biomaterial surface.

2. Materials and methods

2.1. Antibodies, enzymes and cells

Rabbit polyclonal anti-domain V antibody (anti-endorepellin) [23] was provided by Prof Renato Iozzo, Thomas Jefferson University, PA, USA. Mouse monoclonal antibody against heparinase III generated heparan sulfate-stub (clone 3G10) was purchased from Amsbio, Cambridge, MA, USA. Mouse monoclonal antibodies against chondroitin sulfate (clone CS56) was purchased from Sigma Aldrich, St Luis, MO, USA. Biotinylated anti-mouse immunoglobulin and anti-rabbit IgG secondary antibodies and streptavidin-horse radish peroxidase (SA-HRP) were purchased from GE Healthcare, Little Chalfront, UK. Endoglycosidase enzyme heparinase III (EC 4.2.2.8) was purchased from Iduron, Cheshire, UK and chondoitinase ABC (EC 4.2.2.4) from Sigma-Aldrich, St Luis, MO, USA. Human coronary artery endothelial cells (HCAECs) were purchased from Cell Applications, San Diego, CA, USA. All other reagents were purchased from Sigma-Aldrich unless stated otherwise.

2.2. Expression and purification of recombinant perlecan domain V

Domain V of human perlecan was expressed as previously described [21]. Briefly, domain V DNA (2446 bp, exons 79–97) was amplified from human coronary artery endothelial cell (HCAEC) mRNA, cloned into CEFLsec vector with a BM40 signal peptide and transfected into human embryonic kidney 293 (HEK-293) cells using Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific, Waltham, MA, USA). Stably transfected cells were selected using Geneticin. Subsequently, HEK-293 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% (v/v) foetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin and conditioned media routinely collected for domain V purification via anion exchange chromatography on diethylaminoethyl (DEAE) matrix as previously described [24,25]. Domain V was expressed as a proteoglycan decorated with chondroitin sulfate and heparan sulfate glycosaminoglycan chains in a 2:1 ratio.

2.3. Silk solution preparation

Silk fibroin solution was prepared as reported previously [5]. Briefly, pure silk fibroin was extracted from *Bombyx mori* cocoons by degumming the fibers in a sodium carbonate solution (0.02 M) for 30 min to remove sericin. The pure silk fibroin was solubilized in aqueous lithium bromide (9.3 M) at 25% wt/v for 4 h at 60 °C. The solution was dialyzed using SnakeSkin™ dialysis tubing (3500 MWCO, Thermo Fisher Scientific, Waltham, MA, USA) for three days. The concentration of the silk solution was determined by drying a known volume of the solution and massing the remaining solids. This protocol resulted in a 6–8% wt/v silk solution. Silk solution was stored at 4 °C.

2.4. Silk film casting and functionalization with recombinant domain V

Silk solution (2% wt/v in deionized water) was pipetted into 96-well plates (70 μl), 48-well plates (150 μl) or ibidi polymer 8 well μ-slides (Ibidi, Planegg, Germany) (100 μl) and allowed to dry at room temperature. Silk films were water annealed overnight at room temperature as previously described [5].

For passive adsorption, silk films were incubated with recombinant domain V (10 μg/ml in phosphate buffered saline (PBS)) for 2 h at 37 °C. Films were rinsed twice with PBS. For covalent binding, silk films were functionalized as previously described [26]. Briefly, silk films were incubated in 2-(*N*-morpholino)ethanesulfonic acid (MES) buffered saline (0.1 M MES, 0.9% wt/v sodium chloride, pH 6.0) for 30 min at room temperature. The —COOH groups in silk fibroin were activated with 1-ethyl-3-(dimethylaminopropyl) carbodiimide hydrochloride (EDC–HCl)/*N*-hydroxysuccinimide (NHS) solution (0.5 mg/mL of EDC and 0.7 mg/mL of NHS in MES buffered saline, pH 6.0) for 30 min at room temperature, generating amine-reactive NHS-esters on the silk film surface. The activated silk films were washed with MES buffered saline three times and incubated with recombinant domain V (10 μg/ml in MES buffered saline) for 2 h at 37 °C. Films were washed three times with PBS. While covalently functionalised surfaces may contain some degree of passively adsorbed domain V, desorption of domain V was not feasible without inducing conformational changes that will affect subsequent assays.

Selected wells were functionalized with heparinase and/or chondroitinase treated recombinant domain V (to digest heparan sulfate and/or chondroitin sulfate chains from domain V). Recombinant domain V was incubated with heparinase III (0.01 U/ml in PBS) and/or chondroitinase ABC (0.05 U/ml in PBS) for 16 h at 37 °C prior to surface functionalization.

2.5. Surface plasmon resonance analysis

Silk solution (1% wt/v in deionized water, 300 μl) was spin coated onto BIAcore gold chips at 300 rpm for 6 s followed by 2000 rpm for 60 s. The coated chips were water annealed for 24 h as previously described [5]. For covalent binding, the chips were