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The effect of hirudin modification of silk fibroin on cell growth and antithrombogenicity

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ABSTRACT

Thrombus formation remains a particular challenge for small-diameter vascular grafts. In this study, the direct thrombin inhibitor hirudin (Hir) was used to modify silk fibroin films in an attempt to enhance its antithrombogenic properties. Hir was successfully attached to silk fibroin and uniformly distributed in the regenerative material. Hir-modified films showed good cytocompatibility, and supported adhesion and proliferation of fibroblasts (L929), human umbilical vascular endothelial cells (HUVECs) and human aortic smooth muscle cells (HASMCs). Proliferation of HAVSMCs was inhibited by increasing Hir concentration. Activated partial thrombin time (APTT), prothrombin time (PT) and thrombin time (TT) of Hir-modified silk fibroin tubular scaffolds (SFTSs) were all increased markedly compared with fresh rabbit blood, ethanol-treated SFTS and unmodified SFTS, demonstrating the improved antithrombogenicity of SFTSs following modification with Hir.

1. Introduction

Large-diameter (>6 mm) artificial vascular grafts have been very successful in the clinic, but small-diameter (<6 mm) vascular graft applications have proved less successful to date [1]. The primary reasons for their failure are thrombus formation in the short term, and intimal hyperplasia in the long term, which are attributed to the low flow and low blood pressure in the small-diameter vascular lumen, and the thrombogenicity of the blood-contacting surface of synthetic grafts [2]. Upon contact with blood, coagulation occurs on a material surface within minutes, which involves the activation of a cascade of coagulation factors. Once activated, prothrombin in the plasma is converted to thrombin, followed by the formation of insoluble fibrin, which become interconnected and intertwined with platelets, red blood cells and various cellular components, resulting in thrombus block [3–5].

Silk fibroin possesses super biocompatibility and biodegradability, and this material has received much attention due to its potential use in artificial blood vessels [6–9]. To better prevent thrombosis, much research has been conducted into anticoagulant modification of silk fibroin, mainly through heparinization and sulfation. Heparin is a highly sulfated glycosaminoglycan that is used frequently as an anticoagulant in the clinic [10,11]. The antithrombogenicity of silk fibroin can be enhanced by incorporation of heparin [12,13]. Based on the key role played by the NH-SO_3 group in the anticoagulant activity of heparin, researchers have modified silk fibroin by sulfation, and the resultant material has heparin-like anticoagulant activity [14,15]. However, heparin is an indirect thrombin inhibitor, and its anticoagulant activity is dependent on the presence of antithrombin III (AT III) and heparin cofactor II [4]. Thrombocytopenia is another concern in heparin therapy [16–18], and it may be accompanied by severe arterial thrombosis [19–21].

Hirudin, a 65 amino acid peptide, is a potent, specific, and direct thrombin inhibitor that is independent of antithrombin III or cofactors, and that is not inactivated by platelet factor 4 (PF4) [22]. Hirudin has been successfully immobilized on functionalized [2.2]paracyclophane-coated metallic stents, resulting in a less thrombogenic surface [23]. Furthermore, modification of a polyethylene terephthalate (PET) film with Hir decreased platelet adhesion and fibrinogen adherence compared with untreated PET [24]. Incorporation of Hir not only improved anticoagulation properties, but also inhibited the proliferation of smooth muscle cells induced by thrombin, and thus reduced neointimal hyperplasia [25,26].

In present paper, we prepared a Hir modified silk fibroin, and evaluated the cytocompatibility (L929, HUVECs and HASMCs) and antithrombogenic properties (APTT, PT and TT). This study would

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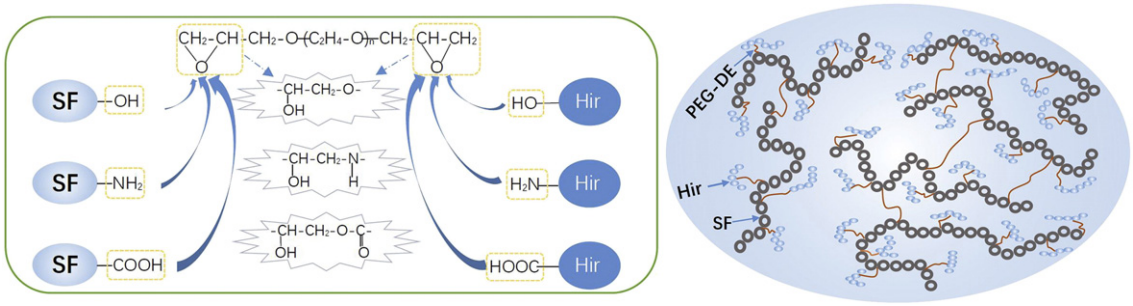

Fig. 1. Illustration of the conjunction between SF and Hir crosslinked by PEG-DE. (A) Principle of chemical reaction; (B) Schematic presentation of modified SF.

provide a method for preparation of anticoagulant materials, expecting to realize application in small-diameter artificial blood vessel transplantation.

2. Materials and methods

2.1. Preparation of regenerated silk fibroin materials

A *Bombyx mori* silk fibroin (SF) aqueous solution was prepared as described previously [27]. Raw silk was first treated three times in 0.06% (w/v) Na_2CO_3 solution at 98–100 °C for 30 min to remove sericin. After drying, degummed silk was dissolved in ternary solvent $CaCl_2 \cdot CH_3CH_2OH \cdot H_2O$ (mole ratio 1:2:8) at 70 ± 2 °C stirred until dissolved. After dialyzing against deionized water at 4 °C for 4 days, the regenerated SF solution was obtained by filtering.

Regenerated SF solution and Hir (Amreso, USA) were then mixed at weight ratios of 10,000:0, 10,000:0.5, 10,000:1, 10,000:2, 10,000:4, and 10,000:6 (based on an approximate molar ratio of SF/Hir) by stirring, and polyethylene glycol diglycidyl ether (PEG-DE, MW500D; Sigma) was slowly added to a SF:PEG-DE weight ratio of 1:0.8. After thorough mixing and degassing, the reaction mixture was cast onto a polyethylene dish and air-dried to generate Hir-modified SF films, which were immersed in deionized water for 3 days to remove uncrosslinked molecules. SF and Hir have abundant —OH, —COOH and —NH_2 groups in their peptide chains, and Hir can be crosslinked to SF when both are exposed to PEG-DE (Fig. 1). Hir-modified SF tubular scaffolds (SFTSs) were obtained by a similar method to that described previously [28]. Briefly, silk braided tubes were first developed by twisting 24 shares of degummed threads of 2 × 20/22 D (diameter units) with 3 × 20/22 D raw silk on a braiding machine (Shanghai, China), and then coated with the reaction mixture containing SF and Hir at the same ratio

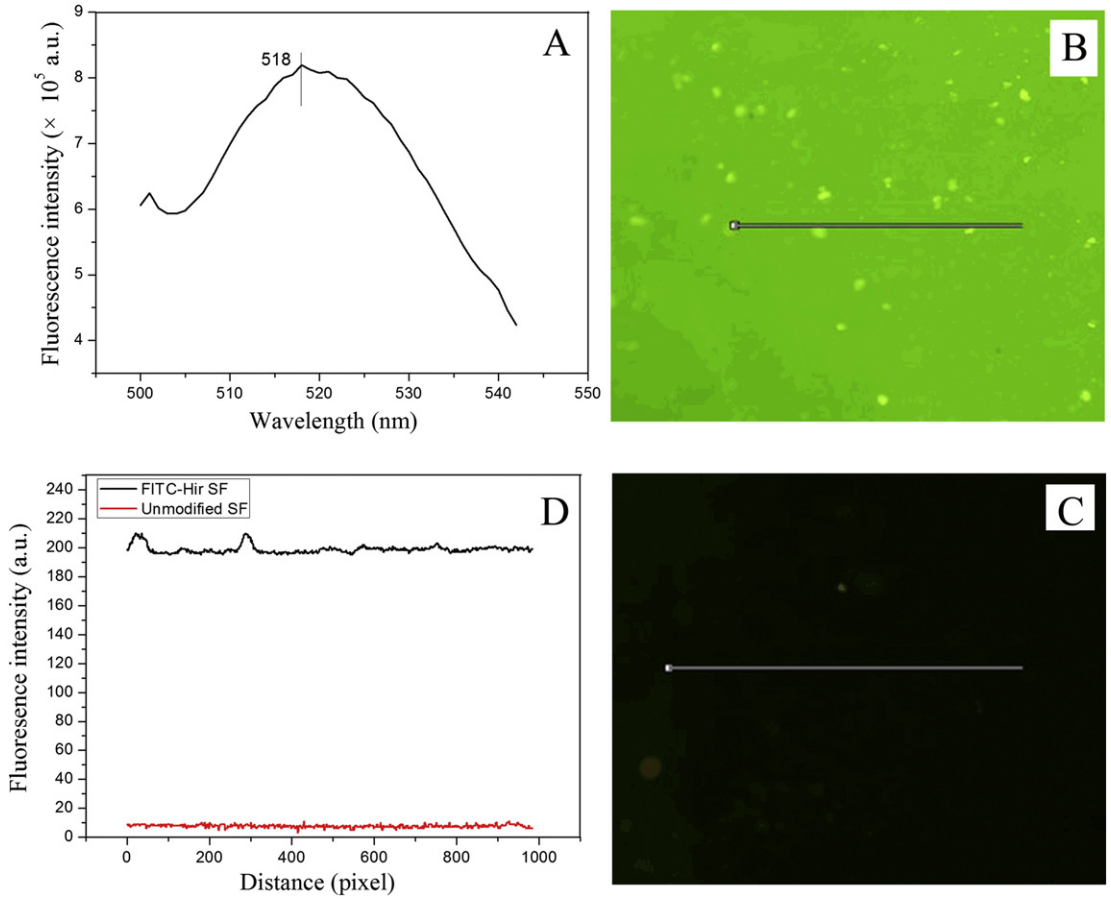

Fig. 2. Distribution of FITC-labeled Hir in SF films. (A) Fluorensence spectra of FITC-Hir/SF; (B) Fluorescence image of Hir-modified SF film; (C) Fluorescence image of unmodified SF film; (D) Fluorensence intensity distribution of FITC-labeled Hir-modified SF film.