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Cooperative control of blood compatibility and re-endothelialization by immobilized heparin and substrate topography

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ABSTRACT

A wide variety of environmental cues provided by the extracellular matrix, including biophysical and biochemical cues, are responsible for vascular cell behavior and function. In particular, substrate topography and surface chemistry have been shown to regulate blood and vascular compatibility individually. The combined impact of chemical and topographic cues on blood and vascular compatibility, and the interplay between these two types of cues, are subjects that are currently being explored. In the present study, a facile polydopamine-mediated approach is introduced for immobilization of heparin on topographically patterned substrates, and the combined effects of these cues on blood compatibility and re-endothelialization are systematically investigated. The results show that immobilized heparin and substrate topography cooperatively modulate anti-coagulation activity, endothelial cell (EC) attachment, proliferation, focal adhesion formation and endothelial marker expression. Meanwhile, the substrate topography is the primary determinant of cell alignment and elongation, driving in vivo-like endothelial organization. Importantly, combining immobilized heparin with substrate topography empowers substantially greater competitive ability of ECs over smooth muscle cells than each cue individually. Moreover, a model is proposed to elucidate the cooperative interplay between immobilized heparin and substrate topography in regulating cell behavior.

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1. Introduction

Coronary artery disease, mostly caused by atherosclerosis, has been a leading cause of death and morbidity in developed countries. Vascular stents or grafts are the most preferred and commonly used prosthesis for treating severe cases of coronary artery disease. However, such interventions are associated with major complications, such as in-stent restenosis, caused by the proliferation of vascular smooth muscle cells (SMCs), and thrombosis, induced by inadequate re-endothelialization on the surface and poor blood compatibility of the vascular stents or grafts. Since the vascular endothelium prevents blood coagulation and SMC proliferation, rapid re-endothelialization is critical to the success of vascular stents or grafts [1]. Several researchers have successfully enhanced re-endothelialization via various surface modification approaches [2–7]. However, the clinical results have been unsatisfactory [8], which may be attributed to the ignorance of competitive growth of endothelial cells (ECs) over SMCs in vivo [9,10]. Therefore, there remains an urgent need for developing multifunctional stent/graft materials, which are able to improve the blood compatibility, favor rapid re-endothelialization and enhance the competitive ability of ECs over SMCs.

In vivo, the vascular endothelium consisting of a monolayer of ECs attaches to the basement membrane (BM), which presents a variety of biophysical and biochemical cues. Recently, there is increasing evidence that the substrate topography, as one of the biophysical cues, is an important surface parameter in determining EC fates and functions [2–4]. In particular, anisotropic topography has been shown to induce an in vivo-like EC elongation and alignment, and potentially improve the EC function, e.g. by enhanced EC migration [2] and an athero-resistant phenotype [11]. Moreover, substrate topography also has a profound influence on the proliferation and differentiation of SMCs [12,13].

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In addition to substrate topography, the biochemical cues presented on the BM also provide instructive signals to ECs. A number of biomolecules have been immobilized onto biomaterial surfaces to promote re-endothelialization [5–7], improve the blood compatibility [14,15] and inhibit SMC proliferation [16]. Heparin, the most widely used anticoagulant drug, has been immobilized on material surfaces to improve blood compatibility by increasing the affinity of antithrombin III to thrombin [14,17,18]. Moreover, the immobilized or released heparin has also been demonstrated to inhibit SMC adhesion and proliferation [19–22]. However, the effects of heparin on EC proliferation are ambiguous. While some groups have demonstrated the enhancement of EC growth induced by heparin [23,24], other groups did not detect significant effects of heparin on EC growth [25,26].

Despite continuing efforts in this field, an ideal system is yet to be proposed that confers multifunctionality in terms of blood compatibility and re-endothelialization. Few studies have modulated blood compatibility and re-endothelialization by integrating both topographic and biochemical cues on biomaterials, and little is known about the cooperative interplay between these two cues. In our previous study, we demonstrated that blood and vascular compatibility can be induced by rationally designed surface topography [27]. Topographic 1 μm grooves induced the maximum EC attachment and proliferation, while concurrently inhibiting SMC growth and platelet activation. In this study, we combine the topographic cue with the biochemical cue via polydopamine-mediated immobilization of heparin on topographically patterned substrates. We also explore the combined effects of substrate topography and immobilized heparin to elucidate their interplay in regulating vascular cell responses, and investigate how this ultimately impacts the blood compatibility and re-endothelialization.

2. Materials and methods

2.1. Fabrication of micropatterned substrates

The micropatterned substrates with anisotropic grooves (ridge = groove = 1 μm, depth = 3.5 μm) were first fabricated on silicon wafers through standard photolithography and deep reactive-ion etching. The patterned silicon substrate was then coated with a thin layer of titanium using a radio frequency sputtering system (Model Explorer 14, Denton Vacuum). A post-sputtering heat treatment was conducted at 700 °C for 1 h under an air flow to transfer the coating layer to the titanium oxide.

2.2. Immobilization of heparin

The heparin immobilization processes are illustrated in Fig. 1. Firstly, primary amine ($-NH_2$) functional groups were introduced onto the substrate surface via co-conjugation of polydopamine (PDA) and poly(ethyleneimine) (PEI). The substrates were immersed in dopamine hydrochloride solution (2 mg ml^{-1}, Sigma–Aldrich, USA) in a tris-(hydroxymethyl) aminomethane (Tris) buffer (10 mM, 15 ml, pH 8.5) at 25 °C for 90 min in an open vessel. After that, PEI solution (20 mg ml^{-1}, average M_w ~1300, 50 wt.% in H_2O, Sigma–Aldrich) in a Tris buffer (10 mM, 15 ml, pH 8.5) was added and surface functionalization was further performed for another 30 min at 25 °C in an open vessel. The functionalized substrate (PEI/PDA) was vigorously washed with deionized (DI) water and blown dry under a weak stream of nitrogen.

Secondly, to immobilize the heparin onto PEI/PDA films, the carboxylic groups of heparin were activated in advance by N-hydroxysuccinimide (NHS) and N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide (EDC), and then were further bound to the PEI/PDA films via their surface $-NH_2$ [18]. Briefly, 2 mg of heparin (sodium salt from porcine intestinal mucosa, potency ⩾180 units mg^{-1}, Sigma–Aldrich) was dissolved in a 20 ml MES (50 mmol l^{-1}, Sigma–Aldrich) buffer solution (pH 5.4) in order to minimize hydrolysis of EDC and then mixed with 20 mg of EDC (Sigma–Aldrich) and 4.8 mg of NHS (Sigma–Aldrich). The PEI/PDA substrates were subsequently immersed in the heparin solution at 25 °C for 12 h. After reaction, the heparin-immobilized substrates (Hep-PEI/PDA) were washed with phosphate-buffered saline (PBS, pH 7.4) and DI water, then blown dry under a weak stream of nitrogen.

2.3. Physiochemical characterization

2.3.1. Film thickness

The thickness of the deposited PEI/PDA film before and after heparin immobilization was measured by a spectroscopic ellipsometer (M-2000V, J.A. Woollam, USA). Δ and Ψ values measured at a wavelength of 370–1000 nm were chosen for data analysis, and the Cauchy model was used to determine the thickness of the deposited PEI/PDA film.

2.3.2. Surface wettability and morphology

The surface wettability of various surfaces was examined by water contact angle (WCA) measurements with a contact angle instrument (Digidrop, France) and applying the sessile drop method. The surface topographies of various surfaces were analyzed by an atomic force microscope (AFM; NanoScope IIIa/Dimension 3100, Digital Instruments, CA). The AFM images were obtained in tapping mode and the root mean square (RMS) was used to evaluate the surface roughness on the basis of a 20 μm × 20 μm scan area. The surface morphologies of various surfaces were observed by scanning electron microscopy (SEM; JSM-6700F, JEOL, Japan).

2.3.3. Zeta potential

The zeta potentials of samples were examined with a commercial electrokinetic analyzer (EKA, Anton Paar GmbH, Graz, Austria). A 0.001 M KCl solution (pH 7.4) was chosen as the electrolyte for the test, which was conducted at room temperature. For each sample, the zeta potential was measured five times and the average value was reported.

2.3.4. Chemical compositions

The quantification of surface elemental composition of samples was measured by X-ray photoelectron spectroscopy (XPS; Kratos, Axis Ultra DLD). A monochromatic Al K_α X-ray was used as the excitation source (hν = 1486.6 eV), running at 15 kV and 150 W. The atomic percentages of the various elements were derived from broad range spectra, using the Al source in a low-resolution mode (pass energy 160 eV), while a pass energy of 20 eV was used for the high-resolution spectra of N1*s* and S2*p*. The C1*s* peak (binding energy 285.0 eV) was used as a reference for charge correction.

2.4. Stability test of PEI/PDA films and immobilized heparin

The PEI/PDA and Hep-PEI/PDA substrates were immersed into PBS solution (pH 7.4) at 37 °C in dynamic state (orbital shakers at 250 rpm) for 3, 7, 15 and 30 days. The surface morphology and atomic concentrations of S2*p* for both untreated and treated substrates were then evaluated, by SEM and XPS, respectively.

2.5. In vitro blood compatibility

The fresh human whole blood used in our experiments was obtained legally from the central blood station of Chengdu, China. Whole blood obtained from healthy human volunteers, who were aspirin-free for a minimum of 2 weeks prior to donating, was

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