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Immobilization of heparin/poly-L-lysine microspheres on medical grade high nitrogen nickel-free austenitic stainless steel surface to improve the biocompatibility and suppress thrombosis

Menghua Li [b,1], Haide Wu [a,b,1], Yi Wang [b], Tieying Yin [b], Hans Gregersen [b], Xiaojuan Zhang [b], Xiaoling Liao [a], Guixue Wang [a,*]

[a] Chongqing Key Laboratory of Nano/Micro Composite Materials and Devices, School of Metallury and Materials Engineering, Chongqing University of Science and Technology, Chongqing, China
[b] Key Laboratory of Biorheological Science and Technology, Ministry of Education, State and Local Joint Engineering Laboratory for Vascular Implants, Bioengineering College, Chongqing University, Chongqing, China

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ABSTRACT

Thrombosis formation, restenosis, and delayed endothelium regeneration continue to be a challenge for coronary artery stent therapy. To improve the hemocompatibility of cardiovascular implants and to selectively direct vascular cell behavior, a novel heparin/poly-L-lysine microsphere was developed and immobilized on a dopamine-coated surface. We chose medical grade high nitrogen nickel-free austenitic stainless steel as the stent material since it has better biocompatibility. The stability and structural characteristics of the microspheres changed with the heparin: poly-L-lysine concentration ratio. Antithrombin III binding was significantly enhanced. Furthermore, for plasma coagulation tests, the activated partial thromboplastin time and thrombin time were prolonged and depended on the heparinfunction. The modified exhibited excellent stability and anticoagulant activity, and efficiently accelerated endothelialization and anticoagulation. This work has potential application for the design of coronary artery stent surfaces tailored for vascular cell behavior.

1. Introduction

For the cardiovascular system, coronary heart disease is the largest threat and the main reason of death and impairment of health [1–2]. The treatment of coronary heart disease includes three categories: drug therapy, surgical treatment and interventional treatment. Percutaneous coronary intervention is an important treatment for coronary heart disease due to small trauma, less pain, and achievement of rapid revascularization due to acute coronary syndrome in case of emergency [3–5]. At present, about 95% of patients have interventional therapy with stents [6]. Stent placement has become the main technology of interventional therapy. SUS316L, Co-Cr and platinum-chromium (Pr-Cr) are the most widely used coronary stent materials [7]. SUS316L and nitinol alloy may release nickel ions, which potentially can be toxic, leading to anaphylactic reaction and cancer [8–9]. Furthermore, SUS316L and nitinol alloy may cause thrombosis due to incompatibility with blood [10–11]. Li et al. [12] examined the biocompatibility of medical grade high nitrogen nickel-free austenitic stainless steel in vitro. The analysis of the mechanical properties and biocompatibility showed that medical grade high nitrogen nickel-free austenitic stainless steel might be used as alternative materials of stent.

Heparin (Hep) is the most commonly used anticoagulant drug and has also been widely used as an anticoagulant coating of material surfaces in contact with blood [13]. The anticoagunt properties of Hep depends on its specific interaction with antithrombin III (AT III), which causes a rapid inactivation of thrombin and other proteases involved in blood clotting [14]. Numerous studies have also shown that Hep can inhibit migration and proliferation of blood vessel cells, especially smooth muscle cells (SMCs) [15]. These in vitro data were further verified in animal testing and in clinical trials, demonstrating that a Hep-coated stent surface helps to prevent thrombosis and intimal hyperplasia [16]. In addition, early in the 1960s, Hep was shown to exhibit excellent performance as an anti-inflammatory drug in various inflammatory disease models [17]. The multifunctional properties of Hep are generally attributed to its interactions with various proteins. To date >100 Hep-binding proteins have been identified [18]. In different experimental setups, Hep may exhibit different or even opposite actions in directing cell behavior. For example, stromal cell-derived factor-1 a (SDF-1 a)-immobilized Hep coating promoted the recruitment of endothelial progenitor cells and SMCs, though the antiproliferante effect of Hep is apparently blocked after binding with the cytokine [19]. Moreover, Hep is considered to harm endothelial cells (ECs) [20]. However, our recent studies suggested that an appropriate Hep dosage selectively enhances

* Corresponding author.
E-mail address: wanggx@cqu.edu.cn (G. Wang).
[1] These authors contributed equally to this work.

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ECs but inhibits SMC proliferation [21–22]. Hence, a heparinized surface with adequate drug density and release kinetics will inhibit thrombosis and restenosis but will not harm the endothelium. Sakiyama-Elbert [23] provided a relatively comprehensive overview of the incorporation of Hep into biomaterials. The most commonly used methods to conjugate Hep seem to be covalent immobilization or electrostatic binding [24]. The former is considered to be stable, but protein binding and anticoagulant activities are reduced due to the use of bioactive carboxylic groups for the immobilization chemistry and the reduced steric accessibility of the immobilized molecule [25]. Electrostatic binding does not harm Hep bioactivity, though burst release remains a major limitation due to the insufficient binding force. In addition, it still appears difficult to control the Hep binding density on the surface.

In this study, the Hep density gradient was constructed on a polydopamine-coated material surface with high stability and bioactivity. It was probed with respect to the behavior of platelets, plasma proteins, as well as ECs. Polydopamine is a mussel-inspired adhesive coating, which has become attractive in the biomaterials field due to its ability to form strong adhesive interactions with materials [26] and with functional biomolecules that contain amine groups [27]. However, Hep is a highly sulfated glycosaminoglycan, rich in hydroxyl, carboxyl and sulfo groups but almost free of amine groups, and hence does not directly bind to a dopamine-coated surface. In this study, a novel Hep immobilizing approach is introduced by covalently binding Hep/poly-L-lysine (PLL) microspheres onto dopamine-coated surface. A similar method has been adopted by Park et al. [28], though the protocol of microsphere preparation and the experimental system they used were very different from our work. As a cationic polymer of amino acids, PLL is commonly used for loading of negatively charged biomolecules, e.g. as gene vectors. Nevertheless, PLL shows a low level of transfection efficiency due to the tight interactions between PLL/DNA complexes [29]. The drawback of PLL in gene transfer could constitute an advantage in terms of immobilizing biomolecules on surfaces. In the present study, amine-rich PLL is mixed with negatively charged Hep to construct Hep/PLL microspheres. Microspheres with different Hep: PLL concentration ratios are subsequently immobilized onto polydopamine (PDA)-coated surfaces. The change in the exposed amine group on different microspheres directly influences the total amount immobilized and thereby creates a Hep density gradient surface. Taking the influence of exposed amine groups into consideration, we have demonstrated that the behavior of platelets and ECs can be selectively regulated over a certain Hep density range.

2. Materials and methods

2.1. HNS preparation

High nitrogen nickel-free stainless steel coupons (HNS, 15 mm diameter, provided by Chongqing Materials Research Institute Co. Ltd) were mirror-polished and ultrasonically cleaned twice with deionized water, acetone and absolute alcohol before use for 5 min each time.

2.2. Hep/PLL microsphere preparation and immobilization

The formation of Hep/PLL microspheres in phosphate buffered saline (PBS) is mainly driven by the electrostatic interaction between negatively charged Hep and positively charged PLL. In general, the particle system is considered moderately stable when the absolute value of the zeta potential is higher than 30 mV. The particle dispersion index (PDI) is another important indicator to evaluate the size distribution of the particles, and a smaller PDI indicated a better uniformity. Preparation of 5 mg ml^{-1}Hep solution and 0.5 mg ml^{-1}PLL solution was done with PBS as solvent under ultrasonic conditions for 5 min to create the suspension of Hep/PLL microspheres. For preparation of PDA coating, the HNS samples were immersed into 2 mg ml^{-1} PDA solution (dissolved in 10 mM Tris buffer, pH 8.5) at 20 °C for 12 h. Then the samples were ultrasonically washed three times with distilled water for 5 min each time, dried in the oven, and marked as one layer. The above operation was repeated twice more, for a total of three PDA layers to be grafted on the sample surfaces. Subsequently, the PDA-coated HNS samples were immersed into prepared Hep/PLL microsphere solutions and incubated at 20 °C for 12 h during gentle shaking. Finally, it was washed three times with distilled water for 5 min and freeze dried to construct Hep/PLL microsphere-immobilized samples.

2.3. Microsphere size and zeta potential analysis

The mean size, PDI, and the zeta potential of the Hep/PLL microspheres dispersed in PBS medium were determined by dynamic light scattering using a Zetasizer Nano-ZS90 (Malvern Ltd., Malvern, UK). Each measurement was repeated three times.

2.4. Characterization of microspheres fixed on dopamine

2.4.1. X-ray photoelectron spectrometer

The surface chemical elemental composition of PDA-coated and Hep/PLL microsphere-immobilized samples was studied by X-ray photoelectron spectroscopy (XPS, Kratos Ltd., UK) on an AXIS His spectrometer with a monochromatic Al Kα X-ray source (1486.6 eV photons, 150 W). The pressure in the chamber was $< 2 \times 10^{-9}$ Torr. The binding energy scale was referenced by setting the C_{1s} peak at 284.6 eV.

2.4.2. Atom force microscopy

The changes in surface topography before and after Hep/PLL microsphere immobilization were characterized by atom force microscopy (AFM, Nanowizard II, JPK Instruments, Berlin, Germany) in contact mode. AFM was done at room temperature and the images were processed by CSPM Imager software.

2.4.3. Water contact angle

Changes in surface hydrophilicity may cause quantitative and qualitative variations of absorbed proteins, which directly influence the biocompatibility of the materials. The static water contact angle on the samples were measured using a DSA100 Mk 2goniometer (Kruss GmbH, Germany) at room temperature. A droplet of deionized water was added to dried sample surfaces. The contact angle was calculated by a circle segment function of the DSA 1.8 software. There were four parallel samples in each group. For each sample, measurements were taken at least at three different sites.

2.4.4. Measurement coating thickness using metaloscope

Focal plane measurements of metaloscope can quantitate the thickness of polylactic acid matrix coatings accurately. Mosaic the coated sample with denture acrylic plates and water, and a longitudinal section on the surface of the sample was polished with a grinding machine. An optical microscope (Metallurgical Microscopy, MM) was used to show the boundary between coating and substrate materials, coating and inlays. The metaloscope images were obtained by charge coupled device,

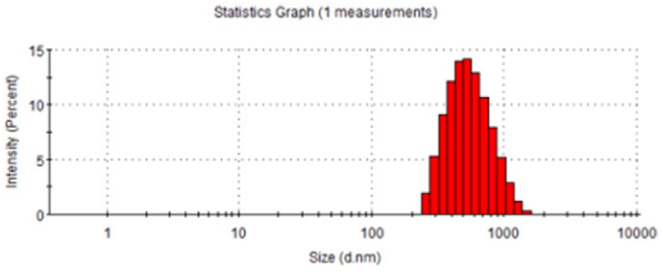

Fig. 1. The distribution of the microsphere size. The mean size of the Hep/PLL particles ranged from 300 nm to 1500 nm.