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Nitric oxide producing coating mimicking endothelium function for multifunctional vascular stents

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ABSTRACT

The continuous release of nitric oxide (NO) by the native endothelium of blood vessels plays a substantial role in the cardiovascular physiology, as it influences important pathways of cardiovascular homeostasis, inhibits vascular smooth muscle cell (VSMC) proliferation, inhibits platelet activation and aggregation, and prevents atherosclerosis. In this study, a NO-catalytic bioactive coating that mimics this endothelium functionality was presented as a hemocompatible coating with potential to improve the biocompatibility of vascular stents. The NO-catalytic bioactive coating was obtained by covalent conjugation of 3,3-diselenodipropionic acid (SeDPA) with glutathione peroxidase (GPx)-like catalytic activity to generate NO from S-nitrosothiols (RSNOs) via specific catalytic reaction. The SeDPA was immobilized to an amine bearing plasma polymerized allylamine (PPAam) surface (SeDPA-PPAam). It showed long-term and continuous ability to catalytically decompose endogenous RSNO and generate NO. The generated NO remarkably increased the cGMP synthesis both in platelets and human umbilical artery smooth muscle cells (HUASMCs). The surface exhibited a remarkable suppression of collagen-induced platelet activation and aggregation. It suppressed the adhesion, proliferation and migration of HUASMCs. Additionally, it was found that the NO catalytic surface significantly enhanced human umbilical vein endothelial cell (HUVEC) adhesion, proliferation and migration. The *in vivo* results indicated that the NO catalytic surface created a favorable microenvironment of competitive growth of HUVECs over HUASMCs for promoting re-endothelialization and reducing restenosis of stents *in vivo*.

1. Introduction

Cardiovascular diseases (CVDs), most frequently owing to atherosclerosis, present worldwide the leading cause of death and disability. Since the first stent implantation into a human coronary artery in 1987 by Sigwart et al. [1], metallic vascular stent has become one of the most common means to treat advanced CVDs. However, the stent expansion easily damages the vessel tissue around the implant. The tissue trauma leads to thrombosis, inflammation, neointimal proliferation and matrix deposition/vessel remodeling [2,3], and results in in-stent restenosis with exaggerated intimal hyperplasia in 20–30% of patients within 3–6 months [4], this in-stent restenosis is a major limitation of this technique.

Improving the biocompatibility of the stents is a central means to reduce in-stent restenosis. Numerous strategies have been explored for this goal. However, current strategies, applied in the commercial stent systems e.g. the clinically widely used drug-eluting stents (DES) [5–7], endothelial progenitor cell-capture (EPC) stents tailoring rapid re-endothelialization [8,9] and new developments as research products [10], mainly focus on improving only one aspect of vascular biocompatibility. Although they show good short-term clinical results, the long-term effects of these stents are far from ideal. Restenosis and late stent thrombosis (LST) remain the two major complications associated with vascular stents. Herein, we conclude that an ideal stent should

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simultaneously exhibit anticoagulant and antiproliferative properties and additionally promote the regeneration of healthy endothelium. Further, the special pathology of the local atherosclerotic lesion is a crucial point, which has to be considered.

However, there are few drugs/biomolecules that possess the profile of action for treating CVDs. A potential candidate is NO, a cell signaling molecule that is constitutively produced in ECs through enzymatic conversion of L-arginine by nitric oxide synthase (NOS). ECs are the primary source of NO in the vascular system. The continuous release of NO is fundamental to maintain cardiovascular homeostasis and regulate vasodilation. In addition, NO is proved to play a crucial role in the prevention of thrombosis, in the inhibition of VSMC proliferation and adhesion, and in the inhibition of leukocyte activation [11–13]. Moreover, NO has been found to have some other important biological functions such as immune response, anticancer and antibacterial effects [14–16]. Important for the current application, NO strongly promotes the healing of atherosclerotic lesions [17,18], and it is a major regulator of EPC in their mobilization, differentiation and function [19]. These unique advantages make NO-releasing stent coatings very promising to meet perfectly the local requirements of an ideal stent concerning the blood vessel wall and the blood phase. Over the past two decades, studies mainly focused on the development of efficient NO-releasing and -generating materials for clinical therapies [20]. For the NO-releasing materials, challenges such as the short half-lives of most NO donors and the uncertainty regarding the safe therapeutic doses of NO for *in vivo* applications limit their commercialization. A stent coating obviously requires a long-term NO delivery, thus exhaustive NO release systems may not be ideal candidates.

There are endogenous NO donors known as RSNO, such as S-nitrosoglutathione (GSNO), S-nitrosocysteine (CysNO) and S-nitrosoalbumin (AlbSNO) in blood [21]. It was found that GPx can catalyze the decomposition of RSNO in the presence of thiols *in vivo* [22]. Organoselenium compounds such as SeDPA and selenocystamine (SeCA) have shown to possess a GPx-like catalytic activity to generate NO from RSNOs [23,24] (The mechanism of the organoselenium for generating NO from RSNOs via a catalytic reaction using glutathione as reducing agent is shown in Scheme S1). Covalent immobilization of such organoselenium compounds on a stent surface may be a feasible way to continuously produce NO, reducing side effects after stent implantation. In order to allow immobilization of the organoselenides on a metal stent surface, in this study, the stent was functionalized with a plasma polymerized allylamine coating first, which provides abundant primary amine groups for coupling SeDPA using carbodiimide chemistry. Despite numerous unique advantages of NO for treating CVDs [11–13,20,25–28], excessive synthesis of NO from exogenous sources might be harmful, as this might imply a greater degree of interactions with superoxide to form peroxynitrites [29]. A safe and rational release rate of NO from exogenous sources must be thereby considered in the design of vascular devices. Therefore, the special aims of this study focused on the investigation of NO catalytic effects and release rates from this NO catalytic coating. Further, the effects of the released-NO on platelet adhesion/activation, the growth behavior of ECs and SMCs and re-endothelialization and anti-restenosis of stent *in vivo* was investigated.

2. Experimental

2.1. Preparation of PPAam coating

The deposition of the plasma polymerized allylamine (PPAam) coating on 316L SS was carried out by a 13.56 MHz pulsed radio frequency (RF) plasma polymerization apparatus, which is described in detail elsewhere [30,31].

2.2. SeDPA conjugation

The 316L SS modified by PPAam coating was immersed into 0.5 mg of SeDPA (Aldrich) per 1 mL of water soluble carbodiimide (WSC) solution (pH 5.4) [31]. After reaction for 12 h, the specimens were washed with PBS and distilled water in sequence. The SeDPA-immobilized PPAam was marked as SeDPA-PPAam.

2.3. Analysis of chemical structure and compositions

Grazing incidence attenuated total reflection Fourier transform infrared spectroscopy (GATR-FTIR) measurement was performed to analyze the chemical structure of the PPAam before and after SeDPA conjugation using Nicolet model 5700 instrument. The surface chemical compositions of the substrates were measured by X-ray photoelectron spectroscopy (XPS, XSAM800, Kratos Ltd, UK). The instrument was equipped with a monochromatic Al Kα (1486.6 eV) X-ray source operated at 12 kV × 15 mA at a pressure of 2×10^{-7} Pa. The C 1s peak (binding energy 284.8 eV) was used as a reference for charge correction.

2.4. Catalytic release of NO

The NO release catalyzed by SeDPA-PPAam was tested using a Griess method [32] and chemiluminescence NO analyzer (NOA) (Seivers 280i, Boulder, CO). For the chemiluminescence method, 316L SS discs (0.5×1 cm^2) modified by SeDPA-PPAam coating was used for NO quantitative analysis. In brief, the NO catalytically produced by samples was purged from the test solution containing RSNO, reducing agent glutathione (GSH) and 500 μM EDTA and transported to the NO analyzer by a stream of N_2 (g). The calculation of the amount of NO generated by samples was based on the calibration curves of the NOA, which was in detail showed elsewhere [23]. In the case of Griess method, the PPAam coating was deposited in a 24-well microtiter plate instead of 316L SS. After immobilization of SeDPA, the Griess reagents and donor containing S-nitrosoacetylpenicillamine (SNAP) or GSNO, GSH and EDTA were simultaneously added, and then the 24-well microtiter plate was immediately analyzed by Microplate absorbance reader at 540 nm. The measurements were corrected for the blank absorbance of the SeDPA-PPAam coating determined before the incubation. In this work, to test the long-term NO-catalytic activity, the SeDPA-PPAam was in advance continuously exposed to the RSNO donor (10 μM SNAP, 10 μM reducing agent glutathione (GSH), 500 μM EDTA) for various periods of time, and this medium was replaced every 12 h.

2.5. Platelet adhesion test and cyclic guanylate monophosphate (cGMP) analysis

Fresh human whole blood was obtained from the central blood station of Chengdu, China, following ethics standards. Whole blood was obtained from healthy human volunteers, who were aspirin-free for a minimum of 2 weeks prior to donation, the blood was anti-coagulated with tri-sodium citrate in a 9:1 volumetric ratio. The analysis was performed within 12 h after the blood donation. PRP was prepared by centrifuging (1500 rpm, 15 min) fresh human whole blood. The amounts of the samples used for statistical analysis were no less than four. All of the tests were performed in duplicate at least.

Due to its chemical instability, little endogenous donor of RSNO is preserved in the final PRP. Therefore, extra donor was added for comparison analysis. In detail, for each sample 1 mL of PRP containing donor (10 μM SNAP, 10 μM GSH) was added. Here, 10 μL collagen-solution (50 μg/mL) was added for the collagen activation assay. Then the specimens were incubated in the PRP or 30 min. The