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Synergetic coordination and catecholamine chemistry for catalytic generation of nitric oxide on vascular stents

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Abstract

The unique advantages of nitric oxide (NO) in cardiovascular disease therapy have driven the development of methods to functionalize cardiovascular stents for local generation of NO. However, current NO-generating materials used for surface engineering stents have limitations such as a complex fabrication process, poor stent adhesion strength, and low control of NO release. Herein, we apply synergetic coordination and catecholamine surface chemistry to develop an adhesive NO-generating coating with a copper-catecholamine framework through a simple, one-step molecule/ion co-assembly process. The copper-catecholic-selenocystamine framework provides glutathione peroxidase (GPx)-like interfacial catalytic activity, which results in long-term, stable, adjustable NO release rates from the coating. The resulting desirable therapeutic dose and release kinetics of NO endow the vascular stent with the ability to simultaneously inhibit platelet activation and smooth muscle cell (SMC) proliferation, and enhances endothelial cell (EC) adhesion, proliferation, and migration in vitro. Vascular stent functionalized by the optimized copper-catecholic-selenocystamine coating significantly suppresses thrombosis, promotes re-endothelialization, and reduces intimal hyperplasia in vivo, and may be promising to address the clinical complications associated with restenosis and late stent thrombosis.

Introduction

Cardiovascular diseases (CVDs) continue to be the leading cause of death and disability all over the world¹. Currently, intervention with cardiovascular stents is the primary means of CVD therapy due to its effectiveness in maintaining vascular patency². However, inevitable vessel wall injury after stenting frequently complicates pathobiology processes associated with leukocyte accumulation, inflammation, smooth muscle cell migration, and proliferation, which ultimately lead to restenosis in addition to the risk of thrombus formation on the foreign surface^{3–5}.

Native endothelium consisting of a monolayer of endothelial cells is an important part of blood vessels and plays a critical role in maintaining homeostasis in the cardiovascular system. Nitric oxide (NO), a unique endogenous signaling molecule in vascular biology, is endogenously produced by the endothelial cells (ECs) through oxidation of L-arginine by nitric oxide synthase (NOS). Continuous release of NO by ECs is crucial for preventing thrombosis⁶, suppressing smooth muscle cell migration and proliferation⁷, inhibiting leukocyte activation⁸, and promoting healing of atherosclerotic lesions⁹. Atherosclerosis, the major reason for cardiovascular diseases, results in the absence of native endothelium and leads to a decrease in NO production. Hence, there is great interest in biomimetic surface modification of cardiovascular stents as delivery vehicles that locally release or generate

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NO to address clinical complications associated with thrombosis and restenosis¹⁰.

The promising application of NO in CVDs has promoted research on efficient generation of NO over the last two decades. Organoselenium moieties and Cu(II) ions, two of the most popular species with glutathione peroxidase (GPx)-like catalytic activity to decompose S-nitrosothiols (RSNO) and known endogenous NO donors, have been used for the design of NO-generating materials^{11,12}. To date, the methods for creating NO-generating materials mainly involve immobilization of organoselenium moieties on polymer matrixes or surfaces of biomaterials with functional groups and doping Cu^{II}-organic ligand complexes into polymer matrixes^{13,14}. However, most existing methods for covalent linking or coating organoselenium species or Cu^{II}-organic ligand complexes on a material surface generally involve tedious multistep processes including pretreatment of the material to introduce the functional groups and complex immobilization or coating procedures. Furthermore, the amount of organoselenium species or Cu^{II}-organic ligand complexes bound to a material is limited by the density of the introduced functional groups on the surface. As a result, the number of catalytic complexes on a cardiovascular stent surface is difficult to accurately control, which leads to an undesirable dose of NO and uncontrollable physiological effects. There are technical challenges in controlling the adhesion strength of polymer coatings containing organoselenium species or Cu^{II}-organic ligand complexes on the stent surface.

Based on the above drawbacks of these strategies for an ideal NO-generating stent coating, herein, we report a one-step molecule/ion co-assembly method to form an adhesive NO-generating coating inspired by the synergetic coordination and catecholamine chemistry found in mussel byssus and adhesive plaque formation processes. In this study, we used 3,4-dihydroxy-L-phenylalanine (Dopa), a very important adhesive amino acid widely present in byssus and adhesive plaques secreted by mussels (Fig. 1a, b), to crosslink selenocystamine (SeCA) and copper ions (Cu²⁺) and fabricate an NO-generating coating, called Cu^{II}-Dopa/SeCA, and bind the coating to the stent surface. Dopa is well known for its specific chemical catechol structure that allows strong interactions with [Fe³⁺] to form [Fe(dopa)₃] coordination complexes, which have been proposed to crosslink Mussel foot protein 1 (mfp-1)¹⁵ in the byssus (Fig. 1c). The formation of [Fe(dopa)₃] coordination complexes in byssus is crucial for mussels to strongly adhere to the rocks¹⁶. Inspired by the byssal cuticle, the coordination chemistry was used to fabricate flexible coatings¹⁷. Additionally, the coexistence of catechol (Dopa) and amine (lysine) groups in the *Mytilus edulis* foot protein 5 (Mefp-5) is regarded as crucial for achieving adhesion to virtually any bulk material surface

(Fig. 1d)¹⁸. In particular, the oxygen of the catechol in an alkaline solution allows Dopa to chemically interact with the amine groups. The resulting catecholamine chemistry allows dopamine and the derivative of Dopa to easily and spontaneously form thin coatings via secondary reactions, creating a variety of functional adlayers and good adhesion to a wide spectrum of materials through oxidative self-polymerization¹⁹. Although coordination chemistry and catecholamine chemistry have been widely investigated for designing functional materials, little attention has been paid to the combination of coordination and catecholamine chemistry, especially for establishing a catalytically active surface. In this study, the NO-generating coating of Cu^{II}-Dopa/SeCA inspired by synergetic coordination and catecholamine chemistry solves the aforementioned challenges. Through a simple one-step “dip-coating” of a cardiovascular stent in an aqueous mixed solution of Dopa, SeCA, and CuCl₂·2H₂O, an adhesive thin Cu^{II}-Dopa/SeCA film was easily coated on a 316L stainless steel (SS, a widespread metal material used for cardiovascular stents) stent. This new method not only simplifies the fabrication process of NO-generating coatings, but also enables the cardiovascular stent to steadily and controllably generate NO in situ.

Material and methods

Materials

Dopa hydrochloride (*M_w* = 189.64 Da, CAS 335081-04-4), selenocystamine hydrochloride (SeCA) (*M_w* = 246.07 Da, CAS 3542-13-0), copper (II) chloride dihydrate (CuCl₂·2H₂O, 99%), and (hydroxymethyl)aminomethane (Tris) (*M_w* = 121.14 Da, CAS 77-86-1) were purchased from Sigma Aldrich. Milli-Q water (18 MΩ, Millipore) was used in all experiments.

Preparation of Cu^{II}-Dopa/SeCA coatings

The Cu^{II}-Dopa/SeCA coating was deposited on a mirror polished 316 L SS substrate (Φ10 mm), 316 L SS foil (0.5 cm × 1 cm), and 316 L SS stent through a one-step dip-coating method by immersing the samples into the reaction solution at room temperature for 24 h. The reaction solution was formulated by dissolving Dopa (0.25 mg/mL), SeCA (0.1 mg/mL), and CuCl₂·2H₂O (defined concentrations from 0 to 50 mg/mL) in Tris buffer (1.2 mg/mL, pH 8.5). Thereafter, all the samples were ultrasonically cleaned with distilled water and dried.

Electron paramagnetic resonance (EPR)

EPR measurements were carried out to investigate the influence of Cu^{II}-Dopa coordination on the formation of the Cu^{II}-Dopa/SeCA framework. EPR spectra were obtained using a Bruker A320 with a microwave bridge (receiver gain, 1 × 10⁵; modulation amplitude, 2 Gauss; microwave power, 20 mW; modulation frequency, 100 kHz).

Fig. 1 Overall view of main reaction pathways involved in Cu^{II} -Dopa/SeCA formation. **a** Mussel byssus and adhesive plaques. **(i)** Schematic illustration of a mussel attached to a substrate surface with adhesive plaques interlinked by byssus; **(ii)** The amino acid sequence of *Mytilus edulis* foot protein 5 (Mefp-5) that is widely present in byssus and adhesive plaques secreted by mussels. The co-existence of two important compounds Dopa and lysine in Mefp-5 is crucial for achieving adhesion to a wide spectrum of materials; **(iii)** $[\text{Fe}(\text{dopa})_3]$ coordination complexes proposed to crosslink mfp-1 in the byssus secreted by mussels. **b** Coordination and catecholamine synergetic surface chemistry for NO-generating coatings inspired by mussels. Dopa and Cu^{2+} with GPx-like catalytic activity to generate NO from RSNOs were chosen to form Cu^{II} -Dopa coordination complexes inspired by $[\text{Fe}(\text{dopa})_3]$ coordination chemistry, and SeCA with a lysine-like crosslinking reaction was further used to link the Dopa- Cu^{II} coordination complexes to form Cu^{II} -Dopa/SeCA polymer-based catecholamine chemistry. Here, SeCA has also been shown to possess GPx-like catalytic activity

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS)

MALDI MS was used to analyze the chemical structure of the Cu^{II}-Dopa/SeCA framework. The detailed process has been reported elsewhere²⁰.

X-ray photoelectron spectroscopy (XPS)

XPS (K-Alpha, Thermo Electron, USA) was performed to detect the surface chemical compositions of the sample. A monochromatic Al K α (1486.6 eV) X-ray source was employed as an excitation source ($h\nu = 1486.6$ eV), which was operated at 12 kV $\times$ 15 mA under a pressure of 3×10^{-7} Pa. The reference for charge correction was the graphitic carbon peak (285 eV).

Thickness measurement

A spectroscopic ellipsometer (M-2000V, J.A. Woollam, USA) was used to measure the thickness of the coating. It was run in the Cauchy model, and Δ and Ψ values were measured in the wavelength range of 370–1000 nm.

Scanning electron microscopy (SEM)

SEM (Quanta 200, FEI, Holland) was used to observe the morphology of the Cu^{II}-Dopa/SeCA-coated 316 L SS vascular stents (1.65 mm $\times$ 18 mm), which was expanded from 1.65 mm to 3.0 mm (diameter) at a pressure of 8 atm by an angioplasty balloon.

Catalytic release of NO

A chemiluminescence NO analyzer (NOA) (Seivers 280i, Boulder, CO) was used to detect the real-time NO release. In detail, the Cu^{II}-Dopa/SeCA coating was deposited on 316 L SS foil (0.5 cm $\times$ 1 cm). After 5 min of baseline calibration of NO level, the sample was plunged into 5 mL of the detecting PBS solution composed of 10 μ M NO donor of S-nitrosoglutathione (GSNO, an endogenous RSNO species) or S-nitro-socysteine (CysNO, an endogenous RSNO species) and 10 μ M glutathione (GSH, an endogenous reducing agent) at 37 °C for approximately 40 min. NO catalytic samples catalyze the decomposition of RSNO to generate NO, which was swept continuously by nitrogen gas (N₂) and transferred to the chemiluminescence detection chamber. In the detection chamber, NO reacts with ozone to produce NO₂^{*} (the excited state of nitrogen dioxide). Then, NO₂^{*} decays with a photon emission (λ between 600–875 nm), which is measured by a photomultiplier tube (PMT), and the signal is proportional to the real-time concentration of NO.

Platelet adhesion test and cyclic guanylate monophosphate (cGMP) analysis

The fresh human venous blood used in this research was obtained from the Chengdu blood center station

following ethics rules. All experiments were carried out within 12 h after the blood donation, and trisodium citrate (4% wt) in a 9:1 volumetric ratio was used here as an anticoagulant.

Platelet rich plasma (PRP) was acquired by centrifugation (1500 r.p.m., 15 min) of fresh human whole-blood. Afterwards, 350 μ L of PRP was dropped onto each sample and incubated at 37 °C in damp air. The NO donor solution (10 μ M GSNO and 10 μ M GSH) was also added to the PRP for comparison analysis. After 30 min, PRP was removed and the samples were washed with saline solution, fixed with glutaraldehyde (2.5%) overnight, and dehydrated and dealcoholized. Finally, the samples were inspected by SEM.

A human cGMP ELISA kit was used to detect the cGMP expression of platelets after being impacted with NO catalyzed by Cu^{II}-Dopa/SeCA. Similar to the platelet adhesion experiment, each sample was incubated in 1 mL of PRP with and without donor supplementation. After 30 min, 100 μ L of 10% Triton X was added to each well and treated with ultrasound. Afterwards, the suspensions were centrifuged at 2500 r.p.m. to isolate the supernatant for analysis.

Human umbilical vein endothelial cell (HUVEC) and human umbilical artery smooth muscle cell (HUASMC) isolation

HUVECs and HUASMCs were obtained from human umbilical vessels. The detailed cell culture processes have been reported elsewhere²¹.

Cell attachment, proliferation, and cGMP analysis

All the in vitro cell (HUVEC and HUASMC) experiments were set as two groups (one with NO donor (10 μ M GSNO and 10 μ M GSH) in the cell media and another without donor in the cell media) to explore the influence of NO generation on cell growth behaviors.

In detail, primary HUVECs and HUASMCs were digested by trypsin (0.25 wt%), seeded on samples with a density of 5×10^4 cells per cm², and then placed in a cell incubator at 37 °C in damp air with 5% CO₂. The NO donor solution was added to the cell media every 4 h. After 2, 24, and 72 h, the samples were sequentially washed with saline solution, fixed with glutaraldehyde (2.5%), and stained with Rhodamine 123. A cell counting kit-8 (CCK-8) was used to detect cell bioactivity after 24 and 72 h of culture.

A human cGMP ELISA kit was used to detect the cGMP expression of HUASMCs impacted with NO catalyzed by Cu^{II}-Dopa/SeCA. Similar to the adhesion experiment, HUASMCs were digested by trypsin (0.25 wt) and then seeded on the samples with a density of 5×10^4 cells per cm². Samples were immersed in a 1 mL cell suspension with and without NO donor (10 μ M GSNO and 10 μ M GSH). After 2 h, 100 μ L of 10% Triton X was

added to each cell well and treated with ultrasound. Afterwards, the cell suspensions were centrifuged at 2500 r.p.m. to isolate the supernatant for analysis.

Co-culture of HUVECs and HUASMCs

To mark the different cells, cell trackers with different colors were used to prelabel HUVECs (green chloromethyl fluorescein diacetate, CMFDA) and HUASMCs (orange chloromethyl trimethyl rhodamine, CMTMR) for 30 min. Then, these two types of cells were digested by trypsin (0.25% wt) and mixed at a ratio of 1:1 in DMEM/F12 medium with 10% fetal bovine serum and a density of 5×10^4 cells per cm^2 . Finally, the mixed cell suspensions were added to the samples and cocultured for 2 and 24 h in a cell incubator at 37 °C in damp air with 5% CO_2 . The cells were observed and photographed using a Leica DMRX fluorescence microscope. The number of adherent cells was counted from at least 12 images by ImageJ.

HUVEC and HUASMC migration assays

The migration assays were performed to investigate the impact of NO generation on the HUVECs and HUASMCs. Cu^{II} -Dopa/SeCA was prepared on 316 L SS foil (0.8 cm $\times$ 2 cm), and both the control and Cu^{II} -Dopa/SeCA-coated foils were bent in the middle to an L-shape. Cells were digested and seeded on one flank of the L-shape with a density of 1×10^5 cells/ cm^2 . After 6 h, all samples were turned over so that another flank of the L-shaped samples was immersed in the cell media and the adherent cells started to migrate. After 24 h, the samples were washed with saline solution, fixed with glutaraldehyde (2.5%), and stained with Rhodamine 123. The migrated cells were observed and photographed using a Leica DMRX fluorescence microscope.

Ex vivo thrombogenicity

All animal handling and surgical procedures were performed in compliance with protocols approved by the Local Ethical Committee and Laboratory Animal Administration Rules of China following the ethical rules. Eight adult New Zealand white rabbits (3.5–4 kg) were used in this experiment. After injecting 30 mg/mL pentobarbital sodium through the ear vein, the rabbit left carotid artery and right external jugular vein were isolated and connected with tubes to form a blood circuit. The uncoated and Cu^{II} -Dopa/SeCA-coated 316 L SS foils (0.8 mm $\times$ 1 mm) were rolled and assembled into tubes. The circuit was stopped after 2 h. The clot in the tube around the specimen was collected, photographed, and weighed. The samples were removed, washed with saline solution, fixed with glutaraldehyde (2.5%) overnight, and dehydrated and dealcoholized. Finally, all the samples were inspected by SEM.

In vivo stent implantation

All processes were performed in agreement with the China Council on Animal Care and Southwest Jiaotong University animal use protocol, in accordance with the ethical rules for experiments on animals. Eight adult New Zealand white rabbits (3.5–4 kg) and sixteen stents were used in this experiment. All rabbits underwent balloon-induced arterial wall injury and were fed a high-fat diet for 2 weeks. After general anesthesia and systemic anticoagulation, the rabbit iliac arteries were isolated, and the uncoated and Cu^{II} -Dopa/SeCA-coated 316 L SS stents were implanted into the left and right iliac arteries, respectively. All animals were fed aspirin for 1 week as postoperative anticoagulation therapy. After 1 and 3 months, the stents and the surrounding vessels were collected and fixed with 10% formaldehyde for 2–3 days. Half of the stents were snipped lengthwise, dehydrated, dealcoholized, and observed by SEM. The other half of the stents were embedded in paraffin and serially cut for further histologic examination.

Results and discussion

Copper-catecholamine framework characterization

To verify that the formation mechanism of the Cu^{II} -Dopa/SeCA framework was based on an ion/molecule coassembly process, electron paramagnetic resonance and matrix-assisted laser desorption ionization mass spectrometry were performed on the product harvested from a reaction solution of Dopa (0.25 mg/mL), $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (0.025 mg/mL), and SeCA (0.1 mg/mL) dissolved in 10 mM Tris buffer (pH 8.5) after 12 h. First, to demonstrate the contribution of the coordination reaction between Dopa and Cu^{2+} to the Cu^{II} -Dopa/SeCA framework, an EPR analysis of the reaction products from Dopa and SeCA with and without the addition of CuCl_2 was first performed (Fig. 2a). For the collected product of Dopa/SeCA without the addition of CuCl_2 , only a radical signal attributed to the oxidation of Dopa was observed (3530 mT)²². In the case of the spectrum of Cu^{II} -Dopa/SeCA, significant signals of Cu^{II} -Dopa coordination complexes were detected (3490–3430 mT), indicating the ion assembly contribution of Cu^{2+} and Dopa to the formation of the Cu^{II} -Dopa/SeCA framework. To more clearly understand the formation mechanism of the Cu^{II} -Dopa/SeCA framework, MALDI MS measurements were carried out. The peak of the $[\text{M} + \text{H}]^+$ ion at 893.5 m/z is clearly present during polymerization (Fig. 2b), indicating the formation of the Cu^{II} -Dopa/SeCA framework via an ion/molecular coassembly process. Additionally, the detected peaks of $[\text{M} + \text{H}]^+$ ions at 444, 665, 686, and 832 m/z revealed the chemistry of Schiff-base formation and Michael addition between the amine and catecholic groups, as well as physical self-assembly between amine and carboxyl groups (Figure S1).

Copper-catecholamine framework for NO-generating coatings

To confirm the feasibility of coordination and catecholamine synergetic surface chemistry for establishing an NO-generating coating, a mixture solution of catechol, Dopa (0.25 mg/mL), SeCA (0.1 mg/mL), and $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ with feed concentrations ranging from 3.125 to 50 $\mu\text{g/mL}$ was employed on a 316 L SS substrate, which is a widespread metal material for vascular stents. After immersion for 12 h, the SS substrates showed a polydopamine-like dark-brown color (Fig. 3a). Ellipsometry analysis revealed that the thickness of Cu^{II} -Dopa/SeCA coatings prepared

with a feed concentration of $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ from 0 to 50 $\mu\text{g/mL}$ ranged from 14 to 23 nm (Fig. 3b). Analysis by X-ray photoelectron spectroscopy revealed the absence of signals specific to the SS substrates (Fig. 3c, Figure S2), indicating complete coverage of the substrates by the coatings. With different feed concentrations of CuCl_2 , coatings with a wide range of atomic compositions of copper and selenium were obtained (Fig. 3d, Table S1), confirming the potential controllability of the Cu^{II} -Dopa/SeCA surface chemistry at the molecular level for vascular stents. Next, the catalytic ability of the Cu^{II} -Dopa/SeCA coatings for NO formation was investigated.

Here, a real-time chemiluminescent assay²³ was used to evaluate the NO-release behavior of the Cu^{II}-Dopa/SeCA coatings on a 316 L SS substrate. Release rates of NO induced by the Cu^{II}-Dopa/SeCA coatings with different feed concentrations of CuCl₂·2H₂O were measured in PBS (pH 7.4) supplemented with 10 μM S-nitrosoglutathione and 10 μM glutathione. As shown in Fig. 4a, real-time monitoring of NO generation from the Cu^{II}-Dopa/SeCA coatings revealed a constant NO release after the samples were immersed in PBS for several minutes, indicating that the coordination and catecholamine synergetic surface chemistry were successful for Cu^{II} chelation and SeCA crosslinking. Additionally, we found that the release rate of NO induced by the Cu^{II}-Dopa/SeCA coatings was highly dependent on the feed concentration of CuCl₂; i.e., for higher concentrations of CuCl₂, higher NO-release rates were obtained (Fig. 4b). More importantly, the Cu^{II}-

Dopa/SeCA coatings exhibited good long-term NO catalytic activity. After continuous exposure to the NO donor in PBS for 1 month, the Cu^{II}-Dopa/SeCA coating retained 44% of the NO-release rate of a native Cu^{II}-Dopa/SeCA coating (Figure S3), which is ascribed to good retention of the Cu content in the coating (Figure S4).

As many endogenous RSNO species (e.g., S-nitrosoglutathione (GSNO), S-nitrosoalbumin (AlbSNO), and S-nitrosocysteine (CysNO)) coexist in fresh blood, we also evaluated the catalytic activity of the Cu^{II}-Dopa/SeCA coating using other RSNO species of CysNO. The chemiluminescent results revealed that an efficient NO-release rate of $5.83 \pm 0.62 \times 10^{-10} \text{ mol} \times \text{cm}^{-2} \times \text{min}^{-1}$ was obtained using 10 μM CysNO (Figure S5A), indicating the effective catalytic activity of our coating using different RSNO species. Moreover, the actual levels of endogenous RSNO in the blood were lower than that used

for NO generation in this study. Therefore, we further evaluated the NO-release behavior of the Cu^{II} -Dopa/SeCA coating using lower levels of GSNO (e.g., 1 μM and 0.1 μM). It was found that the decreased GSNO levels resulted in a visible decrease in NO generation from the Cu^{II} -Dopa/SeCA coating. The NO-release rates were reduced to 2.32 ± 0.37 and $0.56 \pm 0.11 \times 10^{-10} \text{ mol} \times \text{cm}^{-2} \times \text{min}^{-1}$ when 1 μM and 0.1 μM GSNO, respectively, were used (Figure S5B, C), which was lower than that generated by Cu^{II} -Dopa/SeCA using 10 μM GSNO ($4.81 \pm 0.52 \times 10^{-10} \text{ mol} \times \text{cm}^{-2} \times \text{min}^{-1}$). It is noteworthy to mention that patients with endothelial dysfunction produce less NO from the endothelial cell layer lining all blood vessels, and hence, such patients will also likely have lower levels of RSNOs in their blood, which potentially results in a large decrease in the effectiveness of the Cu^{II} -Dopa/SeCA-coated stents. Therefore, the

proposed Cu^{II} -Dopa/SeCA stent coating may not work in every case.

Vascular stents undergo severe deformation during mounting on an angioplasty balloon and expanding when they are deployed. A coating must have sufficient adaptability to resist the deformation behavior of the stent. Herein, a stent analysis was performed as an example to evaluate the mechanical behavior of the Cu^{II} -Dopa/SeCA coating. First, a 316 L SS cardiovascular stent with a size of 1.65 mm $\times$ 18 mm was coated with Cu^{II} -Dopa/SeCA, fabricated using 12.5 $\mu\text{g/mL}$ initial $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, and mounted onto an angioplasty balloon using a stent crimper. Then, the balloon was dilated to 3 mm (diameter) at a pressure of 9 atm for 1 min. After that, the surface morphology of the post-expansion stent was observed by field emission SEM to evaluate the mechanical properties of the Dopa/SeCA coating, as well as the

adhesion strength to stent. The SEM images clearly revealed that the SeCA/Dopa coating remained intact without destruction or peeling from the struts (Figure S6), indicating that Dopa/SeCA coating is sufficiently flexible to follow the deformation of the 316 L SS stent. Taken together, these results indicate that this one-step molecule/ion coassembly method inspired by coordination and catecholamine synergetic surface chemistry clearly outcompetes existing methods for NO-generating coatings.

In vitro evaluation of platelet adhesion and vascular cell growth behavior

The aforementioned properties of Cu^{II} -Dopa/SeCA coatings provide feasibility for further biological applications. NO is widely used to improve the anticoagulant activity and antirestenosis of blood-contacting devices such as tubing^{24,25}, vascular grafts^{26–28}, and cardiovascular stents due to its inhibition of platelet activation and SMC proliferation via the NO-cGMP signaling pathway²⁹. Thus, we first investigated the biological effects of our

NO-generating coatings on platelets and SMCs. Here, for the in vitro tests of platelet adhesion and cell growth behaviors, NO donors GSNO and GSH were supplemented in platelet rich plasma and cell media to simulate the in vivo environment in the cardiovascular system. The results of platelet adhesion revealed that the uncoated and Cu^{II} -Dopa/SeCA-coated 316 L SS all caused severe platelet activation in the control group without an NO donor, whereas significant inhibitory effects on platelet adhesion and activation were found for the Cu^{II} -Dopa/SeCA-coated 316 L SS after supplementation with an NO donor (Fig. 5a). Moreover, the inhibitory effects were enhanced with an increase in the feed concentration of $\text{CuCl}_2 \cdot \text{H}_2\text{O}$ during the preparation of the Cu^{II} -Dopa/SeCA coatings due to the higher cGMP synthesis (Fig. 5b).

Next, the effects of the Cu^{II} -Dopa/SeCA coatings on cell growth behavior were investigated. As expected, the Cu^{II} -Dopa/SeCA coating also showed a remarkable inhibitory effect on HUASMC adhesion by simulating cGMP synthesis in the group with an NO donor (Fig. 6a).

Continuous production of NO also significantly suppressed HUASMC proliferation (Fig. 6b, Figure S7). Despite the enhanced inhibitory effects on platelets and SMCs in the case of a higher NO dose, an excessive dose of NO might be harmful for re-endothelialization in vivo due to the possibility of peroxynitrite formation³⁰. Regeneration of the endothelial layer is a key factor in the

success of stent implantation. Thus, the effect of the Cu^{II} -Dopa/SeCA coatings on EC growth behavior was investigated. We found that if the initial concentration of $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ for the coating on the 316L SS substrate exceeded 12.5 $\mu\text{g/mL}$, the resultant Cu^{II} -Dopa/SeCA coating led to a slight inhibition of human umbilical vein endothelial cell adhesion (Fig. 7a, b) and proliferation

Fig. 7 Adhesion and proliferation of HUVECs incubated in cell culture media with and without the addition of an NO donor (10 μ M GSNO or 10 μ M GSH). **a** Rhodamine staining, **b** cell count (calculated from at least 12 images), and **c** projected area per cell (calculated from at least 100 cells from six different fields) of HUVECs cultured on the samples for 2 h. Proliferation of HUVECs cultured in cell media **c** with and **d** without addition of an NO donor for 24 and 72 h. Data are presented as the mean $\pm$ SD ($n = 4$) and analyzed using one-way ANOVA, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

(Fig. 7c, d and Figure S8). Based on this observation, the Cu^{II} -Dopa/SeCA coatings prepared using 12.5 $\mu\text{g}/\text{mL}$ of $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ were optimized and used as the experimental group for the following biological evaluations.

In recent years, a number of researchers^{31,32} including our group^{10,33,34} have demonstrated that the out-performance of ECs over SMCs endowed by vascular stents is crucial for regeneration of a healthy endothelia layer and implantation success. In this work, the competitive ability of the ECs and SMCs was also tested by coculture of HUVECs and HUASMCs that were labeled with different cell tracker dyes. Competitive cell adhesion and proliferation revealed that the Cu^{II} -Dopa/SeCA coating selectively enhanced HUVEC growth, while suppressing HUASMC adhesion and proliferation on coated 316 L SS, compared with that of the uncoated control, as evidenced by the significant increase in the ratio of HUVECs to HUASMCs in the groups with an NO donor (Figure S9). Additionally, it was found that the Cu^{II} -Dopa/SeCA coating also inhibited HUASMC migration (Figure S10) and enhanced HUVEC migration (Figure S11) in the case of addition of an NO donor.

Ex vivo thrombogenicity

To test the antithrombotic properties of the Cu^{II} -Dopa/SeCA coatings, an ex vivo perfusion experiment was carried out. The 316 L SS foils before and after coating with Cu^{II} -Dopa/SeCA were placed on the luminal

wall of polyvinyl chloride cardiopulmonary perfusion tubes and assembled in a New Zealand white rabbit arteriovenous shunt (Fig. 8a). After 2 h of ex vivo circulation in the arteriovenous shunts supplemented with NO donor, the conditions of thrombosis formation, occlusion induced by thrombosis, and blood flow rates in the coated and uncoated groups were evaluated. As shown in Fig. 8b, c, the uncoated 316 L SS foils induced severe thrombosis and resulted in $85.6 \pm 10.5\%$ occlusion (calculated by measuring the cross-section diameter of the circulating tube) (Fig. 8d), whereas no visible thrombosis and no detectable occlusion were observed in the group of the coated foils. Scanning electron microscopy also confirmed that the coated 316 L SS impressively suppressed platelet activation and fibrin formation (Fig. 8e). On the uncoated 316 L SS, severe thrombi consisting of fibrin, red blood cells, and activated platelets were observed. In contrast, only a small number of platelets with dendritic or even round states were found on the coated 316 L SS. Quantitative analysis of the thrombus weight revealed that the total weight of thrombus harvested from coated 316 L SS foil was 6.6 ± 0.4 mg, which was approximately 8.1% of the weight of the thrombus formed on the uncoated 316 L SS foil (Fig. 8f). Furthermore, blood flow rates in the circuit after 2 h of circulation were tested. Compared with the remarkable reduction in the blood flow rate in the uncoated group ($22.5 \pm 11.3\%$ of the initial rate), the coated group still retained a high flow rate of $87.3 \pm 6.8\%$

Fig. 8 Thrombogenicity of Cu^{II}-Dopa/SeCA-coated 316 L SS foils in a New Zealand white rabbit arteriovenous shunt model. **a** Image of the ex vivo circulation thrombogenicity of rabbits. **b** Cross-sectional photographs of tubing containing 316 L SS foils before and after modification by a Cu^{II}-Dopa/SeCA coating exposed for 2 h to blood flow in a rapid arteriovenous shunt model without heparin. **c** Photograph thrombus formed on 316 L SS foils before and after Cu^{II}-Dopa/SeCA modification. **d** Percent occlusion of the cross-section reveals a significant reduction in occlusion after modification by Cu^{II}-Dopa/SeCA. **e** SEM shows a significant reduction in the activation of platelets and fibrinogen on the Cu^{II}-Dopa/SeCA-coated 316 L SS surface, compared to that of the control 316 L SS surface. **f** Thrombus weight is remarkably reduced in the Cu^{II}-Dopa/SeCA-coated 316 L SS circuit. **g** Cu^{II}-Dopa/SeCA-coated 316 L SS circuit shows obvious improvement in blood flow rate at the end of ex vivo circulation experiments, compared to the control 316 L SS circuit. Data are presented as the mean $\pm$ SD of ex vivo circulation experiments and analyzed using one-way ANOVA, *** p < 0.001

(Fig. 8g). Taken together, these ex vivo results demonstrate that the Cu^{II}-Dopa/SeCA coating inspired by coordination and catecholamine synergetic surface chemistry provides an efficient strategy for surface engineering of vascular stents with antithrombogenic properties.

In vivo cardiovascular stent implantation

To demonstrate the feasibility of our developed Cu^{II}-Dopa/SeCA coating to address the issues of vascular stents associated with restenosis and LST, in vivo stent implantation into iliac arteries of New Zealand white rabbits was further carried out. After 1 and 3 months of implantation, the conditions of re-endothelialization and ISR in uncoated and coated groups were assessed by SEM

and histomorphometric analysis of the implants. As shown in Fig. 9a, severe intimal hyperplasia was found on the surfaces of the uncoated 316 L SS stents, and no cells consistent with an endothelial morphology were observed in the uncoated groups of 316 L SS stents harvested at 1 or 3 months. In contrast, the coated 316 L SS stents were all completely covered by an intact endothelial monolayer, indicating the enhanced re-endothelialization properties of the Cu^{II}-Dopa/SeCA coating. Furthermore, hematoxylin eosin (HE) staining on the cross-sections of the arteries together with the vascular stents was performed to quantitatively evaluate intimal hyperplasia and ISR. As clearly seen in Fig. 9b, the uncoated 316 L SS stents induced significant intimal hyperplasia and ISR, whereas the coated 316 L SS stents impressively inhibited intimal

Fig. 9 Cardiovascular stent modified by Cu^{II}-Dopa/SeCA coating for improved performance. a Re-endothelialization of Cu^{II}-Dopa/SeCA-coated and control 316L SS stents after implantation for 1 and 3 months observed by SEM. **b** The effect of the bare 316 L SS and Cu^{II}-Dopa/SeCA-coated cardiovascular stents on ISR assessed by histomorphometric analysis. **c** Mean neointimal area and **(d)** neointimal stenosis analysis indicated significantly reduced ISR. Data are presented as the mean $\pm$ SD of stent implantation experiments and analyzed using one-way ANOVA, * $p < 0.05$, *** $p < 0.001$

hyperplasia and ISR. Quantitative results from 3 months confirmed that the coated 316 L SS stents decreased the mean neointimal area (Fig. 9c) and neointimal stenosis (Fig. 9d), relative to the uncoated 316 L SS stents by three and two-fold, respectively. Obviously, the developed material-independent synergetic coordination and catecholamine surface chemistry not only opens a new window for efficiently generating therapeutic NO, but also provides an effective strategy to address the clinical issues of cardiovascular stent-associated restenosis and LST.

Continuous release of NO by the endothelium plays a very important physiological role throughout vasculature development^{6–10}. The damage to the native endothelium (e.g., caused by atherosclerosis or stent expansion) results in the loss of endothelium functions, causing decreased NO synthesis. Extra supplementation of NO provided by an implanted vascular stent may be a feasible way to enhance the endothelium functions. Although NO shows unique advantages in the treatment of CVDs, excessive NO provided by exogenous sources might be harmful to cells and tissue by causing a greater degree of interactions with superoxide to form peroxynitrites³⁵, while an insufficient dose of NO is unlikely to meet the need of the therapy. Therefore, a safe and rational dosing of NO from exogenous sources is crucial for the success of vascular stents. Currently, NO-releasing and NO-generating materials, which are two types of materials that can locally release NO, have been explored for engineering the surface of blood-contacting devices (e.g., tubing, vascular grafts, and stents). For NO-releasing materials, a great challenge limiting their commercialization is the short half-lives of most NO donors and the uncertainty regarding the amount of NO generated (burst release of NO in the initial several days after implantation and insufficient dose of NO after implantation for 1–2 weeks) for in vivo applications^{36–39}. In contrast, our reported NO-generating coating of Cu^{II}-Dopa/SeCA demonstrated a relatively long-term, continuous, controllable, and on-demand release of NO. The in vitro NO release showed that the Cu^{II}-Dopa/SeCA coating could retain 44% of the catalytic capacity of a native Cu^{II}-Dopa/SeCA coating (Figure S3), corresponding to an NO-release rate of $2.11 \pm 0.54 \times 10^{-10} \text{ mol} \times \text{cm}^{-2} \times \text{min}^{-1}$. Our in vitro biological evaluations demonstrated that the Cu^{II}-Dopa/SeCA coating prepared using a feed concentration of CuCl₂·2H₂O of 3.125 µg/mL with a release rate of $2.12 \pm 0.13 \times 10^{-10} \text{ mol} \times \text{cm}^{-2} \times \text{min}^{-1}$ (Fig. 4) could effectively inhibit platelet adhesion/activation and SMC proliferation. Additionally, our previous work also revealed that an NO-generating coating subjected to continuous incubation in PBS containing an NO donor for one month retained an approximately $1.8 \times 10^{-10} \text{ mol} \times \text{cm}^{-2} \times \text{min}^{-1}$ release rate of NO. The NO dosing still showed good physiological function for inhibiting platelet activation and SMC

proliferation, while enhancing EC proliferation. This implies that the optimized Cu^{II}-Dopa/SeCA coating in this work could also retain its functions in long-term in vivo applications. As expected, our in vivo stent implantation demonstrated impressively improved anti-thrombogenicity, anti-restenosis, and endothelialization.

Conclusion

In conclusion, we have developed material-independent coordination and catecholamine synergetic surface chemistry for efficient NO-generating coatings with a metal-catecholamine framework. The metal-catecholamine framework was formed using adhesive 3,4-dihydroxy-L-phenylalanine and Cu^{II} ions based on a mussel-inspired molecule/ion coassembly process and equipped with glutathione peroxidase-like activity using organoselenium selenocystamine. The unique metal-catecholamine framework imparts NO-generating coatings with desirable NO catalysis properties, e.g., long-term, stable, and adjustable range of NO production rates. The resulting desirable NO generation endows the NO-generating coatings with excellent antithrombogenic properties and selective abilities to inhibit HUASMC proliferation and enhance HUVEC adhesion, proliferation, and migration in vitro. These unique features help vascular stents significantly suppress restenosis and promote re-endothelialization in vivo. Therefore, such an efficient NO-generating coating with a copper-catecholamine framework provides a facile, safe, and effective means for addressing the major clinical complications of vascular stents.

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Conflict of Interest

The authors declare no conflict of interest.

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