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Polydopamine-mediated long-term elution of the direct thrombin inhibitor bivalirudin from TiO₂ nanotubes for improved vascular biocompatibility†

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Thrombosis and restenosis are two major complications associated with current commercial vascular stents. *In situ* regeneration of a healthy endothelium has been recognized as a promising strategy to address these issues. Numerous strategies have been explored for this goal. However, in most of the cases, they only focused on enhancing endothelial cell growth, ignoring antithrombotic requirements and the competition between smooth muscle cells (SMCs) and endothelial cells (ECs) for their growth. This resulted in non-satisfying clinical results. In this study, we created a multifunctional surface that meets the need of antithrombosis and re-endothelialization. A nanotubular titanium oxide (TiO₂) system has been developed, which elutes the direct thrombin inhibitor, bivalirudin (BVLD); moreover, polydopamine (PDAM) is used to tailor the surface functionality of TiO₂ nanotubes (NTs) for controlling the elution of BVLD. PDAM-functionalized TiO₂ NTs controls the BVLD for more than two months. BVLD eluted from NTs was bioactive and showed a substantial inhibitory effect on thrombin bioactivity, platelet adhesion and activation. In addition, the BVLD-eluting nanotubular TiO₂ system has high selectivity to enhance human umbilical vein endothelial cell (HUVEC) growth, while it inhibits human umbilical artery smooth muscle cell (HUASMC) proliferation. Our design strategy for the BVLD-eluting nanotubular TiO₂ system creates a favorable microenvironment for durable thromboresistance and the promotion of re-endothelialization, and thus it is suitable for the long-term treatment of cardiovascular diseases.

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1. Introduction

Drug-eluting stents (DES), an important tool with the remarkable reduction of the restenosis rate,^{1–3} have been widely used for treating cardiovascular diseases (CVDs). However, the emergence of late stent thrombosis (LST)^{3,4} with fatal outcome seriously limits their clinical application. Current DES mainly focus on the inhibition of vascular SMC proliferation, however at the same time they also suppress EC growth, resulting in a delayed endothelialization.⁵ The bare stent surface poses the risk of LST.

Vascular endothelium consists of a monolayer of endothelial cells (ECs) and it is the natural anticoagulant surface of the blood vessel wall. It is required to maintain vascular

homeostasis and to adjust SMC growth. *In situ* endothelialization is widely considered to be essential for the success of stent implantation.^{6–8} EC growth, including migration, attachment, spreading, survival, proliferation and expression of their typical protein pattern on the vascular stent, plays a crucial role in arterial treatment and endothelium regeneration. Numerous strategies have been explored to stimulate a positive reaction of ECs on a stent surface: Immobilization of bioactive molecules, such as extracellular matrix (ECM) molecules,⁹ cell-adhesive peptides,¹⁰ vascular endothelial growth factor (VEGF),¹¹ proteins,¹² and cell recognition peptides,¹³ are popular means to promote endothelialization. Despite good *in vitro* results or short-term clinical performance, the long-term effects of these stents do not meet the desired ideal effects. Typically, for instance, the anti-CD34 antibody coated stents have been reported to show a significantly enhanced adhesion, *in vitro* proliferation of EPCs, and *in vivo* capture and attachment of EPCs, however, clinical long-term studies revealed that the anti-restenotic effects of the coated stents were not as remarkable as expected.^{14,15} This limited success is attributed to the focus on the recruitment of ECs without considering the subsequent competitive growth of EPCs/ECs with SMCs, therefore resulting in the formation of an imperfect endothelial layer on the stent, ultimately the anti-restenosis effect was suboptimal.

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Ideally, a vascular stent should selectively enhance EC growth, while actively suppress SMC proliferation and show good anticoagulant properties. In several recent years, some researchers, including us, became aware about the importance of the competitive growth of ECs with SMCs on the formation of a pure and healthy endothelium on stents,^{16–20} Some strategies with encouraging results have been explored to direct EC and SMC fate. The strategies mainly focus on the immobilization of bioactive peptides or catechols with EC selectivity, such as Arg-Glu-Asp-Val (REDV) and gallic acid (GA).^{16–18} In addition to bioactive molecules, TiO₂ nanotube coating has been demonstrated to be a promising platform for stent coating with a high selectivity for ECs and suppressing SMCs.^{19,20} TiO₂ nanotubes enhance EC proliferation and the secretion of nitric oxide (NO)²¹ and prostaglandin I₂ (PGI₂),¹⁹ while they suppress SMC proliferation and promote the expression of smooth muscle α -actin.¹⁹ They also show low immunogenicity through eliciting minimal levels of monocyte activation and cytokine secretion.²² However, these surfaces face a common issue, insufficient anticoagulant properties. Smith *et al.*²³ have reported severe blood clotting at TiO₂ nanotube coatings. Further developments are needed to solve this obstacle for an ideal stent platform. It is noted that in addition to the desired property of vascular cell selectivity, TiO₂ nanotube coating is also an ideal drug-eluting system.^{24,25} The elution of anticoagulant reagents/drugs from TiO₂ nanotube coating may be a possible process to address the issue of poor hemocompatibility of TiO₂ nanotube coating.

In this work, for the development of an ideal TiO₂ nanotube coating-based stent platform, the direct thrombin inhibitor, bivalirudin (BVLD, 2180 Da, Fig. S1†) was chosen as eluted anticoagulant due to its excellent anticoagulant property (good inhibition for both circulating and clot-bound thrombin, as well as inhibition in thrombin-mediated platelet activation and aggregation),^{26–28} safety (no risk for heparin induced thrombocytopenia HIT II)²⁹ and its appropriate hydrodynamic radius (<10 nm) for loading into the TiO₂ nanotubes (70–90 nm).

Stenting is associated with a complex pathobiological response. The vessel wall injury at stent implantation causes thrombus formation, followed by leukocyte accumulation, inflammation, SMC migration and vessel wall remodeling. The healing usually involves a long process. Therefore, to be clinically useful, drug release from stents should be in sufficient amounts and last for an extended period of time. Herein, we present a versatile route for increasing the loading capacity of BVLD and extending the duration of BVLD release based on mussel-inspired surface chemistry. Mussel-inspired polydopamine (PDAM) coating has drawn considerable attention due to its good adhesion strength virtually for all the types of surfaces and secondary reactivity for anchoring or adsorbing biomolecules.³⁰ In this investigation, a thin ad-layer of PDAM was deposited on TiO₂ nanotube arrays to tailor its surface functionalities for moderating a controllable loading and release of BVLD (Fig. 1). Systemic biocompatibility evaluation of platelet adhesion and growth behavior of ECs and SMCs were further performed.

2. Experimental

2.1 Fabrication and characterization of TiO₂ nanotube arrays

TiO₂ nanotube arrays were fabricated by anodic oxidation in an electrolytic solution mixture of glycerol and water (4 : 1, v/v) containing 0.5 wt% NH₄F and 0.35 wt% NaCl. A titanium foil (0.25 mm thick) was anodized at a constant direct current voltage of 25 V for 4 h. After cleaning the as-prepared nanotube arrays by distilled water, they were annealed in air at a temperature of 450 °C for 6 h. The obtained TiO₂ nanotube arrays were further cleaned by ethanol and distilled water before they were used in surface analysis and sterilized with 75% ethanol and UV prior to their use in cell experiments. Field emission SEM (JSM-6390, JEOL, Japan), X-ray diffractometry (XRD, X'Pert Pro MPD), XPS (Perkin Elmer 16PC) and Drop Shape Analysis DSA100 (Krüss, Hamburg, Germany) were used to characterize the surface morphology, crystal structure, surface elemental compositions and WCA of the samples, respectively.

2.2 PDAM surface modification

The TiO₂ nanotube arrays were in advance treated by oxygen-plasma for 1 min. Subsequently, the specimens were ultrasonically immersed into 0.5 mg mL^{−1} dopamine hydrochloride Tris-based buffer solution (1.2 mg mL^{−1}, pH 8.5) for 12 h at a constant temperature of 20 °C. The PDAM-modified TiO₂ nanotube arrays were then ultrasonically cleaned in distilled water and dried before use.

2.3 BVLD loading and release

1 cm × 1 cm TiO₂ nanotube arrays, before and after PDAM ad-layer modification and the control flat Ti pieces were treated with oxygen plasma for 1 min before BVLD loading. Subsequently, 30 μ L of BVLD (10 mg mL^{−1}) in deionized (DI) water was applied to each surface and then exposed to ultrasound for several seconds to enhance wetting and remove air bubbles. About 30 μ L BVLD solution was then repeatedly added to each surface. The specimens were air dried in a chemical hood, and the loading procedure was repeated twice for BVLD. The loaded specimens were respectively immersed into phosphate buffer solution (PBS, pH 7.4) and DI water for removing the unbound BVLD.

The BVLD release from the abovementioned samples was determined in PBS (pH 7.4). The samples were immersed in airtight centrifuge tubes with 6 mL PBS and incubated at 37 °C under constant rotation (90–95 rpm). Samples of the elution medium were removed at specified intervals by removing half volume of PBS and replacing it with the equal volume of fresh PBS. The BVLD-loading content was measured photometrically at 274 nm using a UV-vis Spectrophotometer (UV-2550, Shimadzu, Japan) and calibrated with standards of defined concentrations. The total amount of BVLD loaded to the samples was determined by ultrasonic elution to the medium. The eluted medium was taken by removing the entire volume of 3 mL PBS after ultrasonic treatment (3 times, 5 min) and

Fig. 1 Scheme of the procedure for BVLD loading: (A) bare TiO₂ nanotube arrays, (B) surface modification of TiO₂ nanotube arrays by PDAM, (C) BVLD loading and (D) the possible coupling manners of loaded BVLD on PDAM-modified TiO₂ nanotube arrays.

replacing it with fresh PBS every time. The procedure was repeated until there was no absorbance observed for the eluted medium at 274 nm.

2.4 Thrombin adsorption and activity

For the test of thrombin adsorption, a chromogenic method was used.³¹ In brief, the surfaces were equilibrated with Tris-BSA buffer (50 mM Tris, 100 mM NaCl, 0.2% BSA, pH 7.4) for 30 min to avoid non-specific adsorption. After removing Tris-BSA buffer from the samples, 60 μ L of 0.2 mg mL⁻¹ thrombin in Tris-BSA buffer was placed on each surface. After incubation for 1 h at 37 $^{\circ}$ C, 10 μ L supernatant was taken and diluted with 90 μ L Tris-BSA buffer and it was then added to 100 μ L chromogenic substrate (250 μ M S-2238 (H-D-Phe-Pip-Arg-pNA $\cdot$ 2HCl in Tris-BSA)). The thrombin activity was then determined at 405 nm in a kinetic chromogenic assay with S2238 substrate against standards of defined thrombin concentrations. The amount of thrombin adsorbed on each sample is considered to be equal to thrombin consumption in the supernatant.

The chromogenic method was also used to test thrombin activity. The detailed method has been previously described.³²

2.5 Platelet adhesion test

Platelet rich plasma (PRP) was prepared by centrifuging (1500 rpm, 15 min) fresh human whole blood. 50 μ L of fresh PRP was distributed on the samples (1 cm $\times$ 1 cm) and incubated for 2 h at 37 $^{\circ}$ C in humidified air. After washing with 0.9% NaCl solution, they were fixed using 2.5% glutaraldehyde solution for 12 h, and then again washed with 0.9% NaCl. After that the specimens were step-by-step dehydrated in 50, 75, 90 and 100% ethanol solutions and were then dealcoholized in 50, 75, 90 and 100% isoamyl acetate solutions. After critical point drying, the obtained samples were inspected by SEM. Immunofluorescence of platelet P-selectin was used for the evaluation of platelet

activation; the detailed experimental procedure has been described elsewhere.³²

2.6 HUVEC and HUASMC culture

HUVECs and HUASMCs were derived from human umbilical vein and arteries. The detailed processes of cell culture are described elsewhere.³²

2.7 HUASMC adhesion and proliferation

In brief, HUASMCs were seeded on the specimen surfaces at a density of 5×10^4 cells per cm² for 2 h, 1, 3 and 5 days respectively. Here, rhodamine 123 staining was performed to analyze the morphology of the cells. Cell Counting Kit-8 (CCK-8) was used to evaluate cell proliferation, as described in detail elsewhere.³³

2.8 HUVEC adhesion and proliferation

Actin immunostaining of HUVECs was used for analyzing the cell morphology, and Cell Counting Kit-8 (CCK-8) was used to measure cell proliferation, the cell culture time was 2 h, 1 and 3 days, respectively. The cell tests and statistical analysis of cell morphology are described in detail elsewhere.³²

2.9 NO release of adherent HUVECs

NO release by ECs is a very important indicator of the function of ECs. Here, a common method of Griess reagent was used to determine the release level of NO produced by HUVECs. In brief, the specimens were first cultured in a medium containing HUVECs at high density of 5×10^5 cells per cm² for 8 h to rapidly form a dense cell monolayer that mimicked the natural endothelium. Subsequently, the specimens with a confluent cell monolayer were transferred to new culture plates, and cultured for 6, 12 and 24 h, respectively. Finally, the cell culture medium

was collected, centrifuged and the supernatant was mixed with an equal volume of Griess reagent (40 mg mL^{-1} , Sigma) and measured at 450 nm. The detailed experimental procedure is described elsewhere.³²

2.10 Migration assays of HUVECs and HUASMCs

The enhancement in EC migration and the inhibition in SMC migration are crucial for *in situ* regeneration of a pure and healthy EC layer on a vascular stent. In this work, both HUVEC and HUASMC migration assays were performed. As the commonly used migration assay based on the scarification of the endothelial layer damages the soft coating, a new method was developed in our previous work.³² Briefly, cells were seeded only on one arm of an L-shaped sample, such that a monolayer with a sharply defined edge was achieved. After that the sample was turned and the growth of the cells on the other arm of the sample was documented. Thus, this method was used to determine the HUASMC and HUVEC migration.

2.11 Co-culture of HUVECs and HASMCs

The co-culture of HUVECs and HASMCs was performed to evaluate the competitive adhesion behaviors of the two cell types. Before the preparation of culture, HUVECs were labeled with CellTracker Green CMFDA, and HASMCs were labeled with Orange CMTMR according to the product instructions (Molecular Probes, USA). The HUVECs and HASMCs suspensions were then mixed in a volume ratio of 1 : 1 and the cells were seeded at a density of 5×10^4 cells per cm^2 . The competitive cell adhesion was examined after 2 h incubation. The detailed experimental procedure is described elsewhere.¹⁸

2.12 Statistical analysis

All cell experiments were performed at least in duplicate, and quantified with at least 4 replicates. The data were presented as mean $\pm$ standard deviation (SD) and statistically analyzed by a one-way ANOVA using the IBM SPSS Statistics 19 software. The value of p less than 0.05 represents statistically significant difference.

3. Results and discussion

Previous studies have demonstrated that anatase TiO_2 nanotube arrays with a diameter of 70–90 nm and length of about

1 μm are beneficial for cell growth and controlled drug release.^{20,22,24,25} In this work, TiO_2 nanotube arrays with this style (Fig. 2A and B and S2†) were fabricated by anodic oxidation in a NH_4F aqueous solution. PDAM coating was achieved by immersion in a dopamine HCl solution. To evaluate the influence of PDAM deposition on the surface morphology of the TiO_2 nanotube arrays, scanning electron microscope (SEM) characterization was performed. The SEM images (Fig. 2C) demonstrate the obvious morphological changes of the TiO_2 nanotube arrays after the deposition of PDAM, which typically embodied a reduced pore diameter of the nanotubes and the adhesion of some polymer particles.

To demonstrate the successful modification of TiO_2 nanotube arrays by PDAM, water contact angle (WCA) and X-ray photoelectron spectroscopy (XPS) measurements were carried out. As shown in Fig. S3† and Table S1,† the deposition of PDAM resulted in significant changes both in WCA and the chemical composition of TiO_2 nanotube arrays. As-deposited PDAM on any flat substrate material shows WCA close to 50° .^{30,34,35} The deposition of PDAM on the TiO_2 nanotube arrays increased the WCA from close to 0° to about 30° (Fig. S3†). The XPS analysis revealed the substantial decrease of the titanium signal specific to TiO_2 nanotube arrays and the presence of characteristic element nitrogen of dopamine (Table S1†). This indicates the formation of a thin polydopamine film on the TiO_2 nanotube arrays. In addition, loading with BVLD also led to significant changes in both WCA (Fig. S3†) and chemical composition (Table S1†).

In order to understand the influence of the PDAM ad-layer on the elution kinetics, the control TiO_2 nanotube arrays (NTs), PDAM-modified TiO_2 nanotube arrays (PDAM/NTs) as well as flat titanium (Flat Ti) were loaded with BVLD by immersion in a BVLD solution, using ultrasound energy for better diffusion into the pores. We found that the control NTs showed good loading capacity of BVLD (Table S2†). The PDAM ad-layer modification to NTs led to a significant 162% increase in total mass of BVLD loading (Table S2†), the total mass of BVLD loaded on PDAM/NTs reached $276.6 \pm 9.8 \mu\text{g cm}^{-2}$. The increased mass of BVLD loaded on the NTs is attributed to the PDAM ad-layer. In addition, BVLD that was loaded into the large gaps between the tubes of the control NTs was appearing to be stripped off during the washing step as compared with the PDAM/NTs. Moreover, the smaller diameter of the nanotube of PDAM/NTs and the interactions of the phenolic hydroxyl group/amine groups of

Fig. 2 SEM images of TiO_2 nanotubes. (A) The top surface showing the TiO_2 nanotube structure; (B) typical cross-sectional image of self-supporting TiO_2 nanotube layer; (C) surface topology structure of TiO_2 nanotube after deposition of PDAM coating.

PDAM/NTs and loaded-BVLD (Fig. 1) may explain the lower BVLD loss during the rinsing step.

The BVLD release characteristics of the Flat Ti, NTs and PDAM/NTs are further compared by photometric determination of the released peptide (Fig. 3). Obviously, the release profiles are significantly different for the three sample types. The PDAM/NTs provided not only more BVLD elution (Fig. 3A) but also a considerably longer release profile (Fig. 3B) as compared with the NTs and flat Ti. Moreover, the PDAM/NTs released the lowest fraction of the loaded BVLD (Fig. 3B). The half-life of BVLD release ($t_{1/2}$) of the Flat Ti and NTs was only about 10 days, whereas the $t_{1/2}$ of the PDAM/NTs reached 35 days. After continuous elution for 70 days, the control NTs released about 84.5% of loaded BVLD, while only 70.5% of loaded BVLD was released from PDAM/NTs. This revealed that the reduced pore diameter of the nanotubes after PDAM ad-layer formation and the existence of the phenolic hydroxyl and amine groups of PDAM coating play an important role not only in increasing BVLD loading but also in prolonging the BVLD release. These unique advantages of PDAM/NTs in the durable and controllable elution of BVLD provide the potential for application in long-term implanted devices.

Fig. 3 BVLD elution for flat Ti, NTs and PDAM/NTs (the size of sample: 1 cm × 1 cm, $n = 8$) after loading with BVLD and brief washing of unbound substance. (A) Absolute BVLD release; PDAM/NTs release highest amount of BVLD, and there is still ongoing release after 70 days. (B) BVLD release in terms of the percentage of the loaded amount. After 70 days, the PDAM/NT system has released only 60% of the initially loaded drug.

Thrombin plays an essential and important role in the coagulation process. Thrombin not only activates the coagulation factors XI, VIII, V and I (fibrinogen) in the blood coagulation pathway, but as part of its activity thrombin also promotes platelet activation and aggregation *via* the activation of protease-activated receptors on the cell membrane of the platelet. The direct thrombin inhibitor bivalirudin directly inhibits thrombin by specifically binding to both the anion-binding exosite 1 and the catalytic site of both fibrin-bound and circulating thrombin.²⁷ Immobilization of BVLD on a material was demonstrated to be a useful way to improve the hemocompatibility in our previous works.^{31,36} It has been found that the anticoagulant capacity is closely associated with the retention of amount and bioactivity of the immobilized BVLD. Thus, the effects of BVLD loading on NTs (BVLD-NTs) and PDAM/NTs (BVLD-PDAM/NTs) for thrombin adsorption and activity were investigated. Compared with flat Ti, NTs showed increase in the thrombin adsorption (Fig. S4A†) and inhibited the activity of the adsorbed thrombin (Fig. S4B†). The modification of PDAM to NTs further promoted thrombin adsorption, and the activity of adsorbed thrombin was better maintained on its surface. The loading of BVLD either to control NTs or PDAM/NTs resulted in a substantial enhancement in thrombin adsorption and suppression in thrombin activity. The thrombin activity on the different surfaces was normalized to the quantity of thrombin present on these surfaces to obtain a specific thrombin activity (Fig. 4). Although PDAM coating on NTs clearly accelerated thrombin activation, the further loading of BVLD significantly inhibited the thrombin activity and caused the lowest thrombin activity per amount of adsorbed thrombin. This indicates that BVLD loaded either on the control NTs or the PDAM/NTs well retained its functionality as thrombin inhibitor. Moreover, the significantly prolonged clotting time (Fig. S5†) confirmed the successful loading of BVLD and the excellently maintained bioactivity.

The substantial inhibition of the thrombin activity by BVLD-NTs and BVLD-PDAM/NTs also suggests potential effects on preventing Fg activation and platelet activation/aggregation. To

Fig. 4 Thrombin activity on the surfaces, normalized to the quantity of thrombin present; ratio between activity (absorbance) and adsorbed thrombin ($\mu\text{g cm}^{-2}$) (the size of sample: 1 cm × 1 cm, $n = 4$). Data presented as mean $\pm$ SD and analyzed using a one-way ANOVA, * $p < 0.05$, *** $p < 0.001$.

attest this assumption, Fg adsorption and platelet adhesion tests were carried out. As expected, both BVLD-NTs and BVLD-PDAM/NTs remarkably suppressed Fg activation (Fig. S6†). SEM images of platelet morphology also revealed a significant inhibitory effect of BVLD-NTs and BVLD-PDAM/NTs not only on activation and aggregation but also on the adhesion of platelets (Fig. 5A). As shown in Fig. 5, on the surfaces of the Flat Ti, most of the adherent platelets were in an activated state with pseudopodia. Although the formation of NTs and further modification by PDAM ad-layer did not make a significant effect on the number of adherent platelets (Fig. 5C), it stimulated the further activation of the platelets (Fig. 5D) as evidenced by P-selectin stainings (Fig. 5B). However, the loading of BVLD to NTs led to a substantial decrease of both platelet adhesion and activation. Importantly, the BVLD-PDAM/NTs showed considerably stronger inhibition in platelet adhesion and activation due to the increased amount of loaded BVLD. The platelets adhered on BVLD-PDAM/NTs surface remained spherical and separated without pseudopodia, which can be visually confirmed by P-selectin stainings shown in Fig. 5B.

In the early stage of stent implantation, in addition to thromboresistance, an anti-proliferative property against smooth muscle cells is crucial to inhibit in-stent stenosis. Therefore, the growth behavior of VSMCs on BVLD-PDAM/NTs was investigated. Fig. 6A shows the typical fluorescence microscopic images of HUASMCs grown on different surfaces for 2 h, 1, 3 and 5 days. 2 h of cell culture revealed a significant reduction in the number of HUASMCs attached on the NTs (45.9%) compared with the flat Ti (Fig. 6B). In addition, the HUASMCs attached on the NTs expressed the resting spherical state and showed considerably smaller spreading areas than on the flat Ti. With increase in culture time, although HUASMCs proliferated on both flat Ti and NTs, the proliferation rate of HUASMCs on NTs was much lower than on the flat Ti (Fig. 6C). These observations indicate a substantial inhibitory effect of NTs on HUASMC attachment and spreading, cytoskeleton development and proliferation.¹⁹ The loading of BVLD to the NTs led to a further decrease in the number of adherent HUASMCs, while there was no significant effect on cell morphology and proliferation. The introduction of PDAM

Fig. 5 (A) Morphology of adherent platelets on the surfaces of various samples observed using SEM; (B) representative images of P-selectin staining, showing activated platelets labeled with a green fluorescent dye. (C) The number of the adhered platelets and (D) the ratio of the activated platelets on the flat Ti, NTs, PDAM/NTs, BVLD-NTs and BVLD-PDAM/NTs (the size of sample: $1\text{ cm} \times 1\text{ cm}$, $n = 4$) are calculated from at least 12 SEM images. Data presented as mean $\pm$ SD and analyzed using a one-way ANOVA, $**p < 0.01$, $***p < 0.001$.

Fig. 6 (A) Fluorescent images of HUASMCs on Flat Ti, NTs, PDAM/NTs, BVLD-NTs and BVLD-PDAM/NTs surfaces (the size of sample: 1 cm × 1 cm, $n = 4$) after 2 h, 1, 3 and 5 days culture. (B) Amounts of HUASMCs attached onto Flat Ti, NTs, PDAM/NTs, BVLD-NTs and BVLD-PDAM/NTs surfaces after 2 h culture, calculated from at least 12 images. (C) Proliferation of HUASMCs grown on Flat Ti, NTs, PDAM/NTs, BVLD-NTs and BVLD-PDAM/NTs surfaces after 1, 3 and 5 days culture. Data presented as mean $\pm$ SD and analyzed using a one-way ANOVA, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

ad-layer at the NT coating stimulated HUASMC spreading, which resulted in the enhanced proliferation of HUASMCs. This may be attributed to the decreased diameter of the nanotubes or the chemical cues of the PDAM ad-layer. The loading of BVLD on PDAM/NTs reduced the HUASMC attachment and spreading area. This suggests that BVLD facilitates the maintenance of the differentiated state of HUASMCs, thus reinforcing the non-proliferative phenotype. The reduced HUASMC proliferation indirectly confirmed this deduction (Fig. 6C).

The vessel tissue around the implant region is easily damaged when the stent is expanded. This tissue trauma leads to SMC migration and proliferation, and results in in-stent restenosis. Therefore, the inhibition of SMC migration is equally crucial in the early stage of stent implantation in the form of SMC proliferation. Therefore, a test of SMC migration was performed. As shown in Fig. 7, the NTs whether modified by PDAM or loaded with BVLD strongly inhibited HUASMC migration compared to flat Ti. Both the migration distance and

Fig. 7 Migration of HUASMCs on the Flat Ti, NTs, PDAM/NTs, BVLD-NTs and BVLD-PDAM/NTs surfaces (the size of sample: 0.8 cm × 2.2 cm, $n = 6$) after a culture of 1 day. (B) The migration distance of migrated cells was calculated from at least 12 images. Data presented as mean ± SD and analyzed using a one-way ANOVA, $**p < 0.01$, $***p < 0.001$.

Fig. 8 (A) Cytoskeletal actin stains of HUVECs grown on Flat Ti, NTs, PDAM/NTs, BVLD-NTs and BVLD-PDAM/NTs surfaces (the size of sample: 1 cm × 1 cm, $n = 4$) after culture of 2 h, 1 and 3 days. (B) Amounts of HUVECs attached onto Flat Ti, NTs, PDAM/NTs, BVLD-NTs and BVLD-PDAM/NTs surfaces after the culture of 2 h; calculated from at least 12 images. (C) Proliferation of HUVECs grown on Flat Ti, NTs, PDAM/NTs, BVLD-NTs and BVLD-PDAM/NTs surfaces after cultures of 1 and 3 days. Data presented as mean ± SD and analyzed using a one-way ANOVA, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

density of the migrated HUASMCs on the control NTs, PDAM/NTs, BVLD-NTs and BVLD-PDAM/NTs were considerably lower than on the flat Ti, suggesting a huge anti-restenotic potential.

Enhanced regeneration of a healthy endothelium is one of the most important functionalities, which determines the success of a vascular stent. The enhanced recruitment, migration, adhesion, spreading, viability, proliferation and the expression of specific proteins and mediators by the ECs play the important roles in endothelium regeneration. Thus, it is very important to understand the interactions between the ECs and the surface of an implanted material. A eukaryotic cell adheres to a substrate generally during the process of attachment, spreading, cytoskeleton development, and then proliferation.³⁷ Therefore, the growth behavior of ECs on these materials was systemically investigated.

Fig. 8A shows the typical fluorescence microscopic images of HUVECs. First, we studied the short-time attachment of HUVECs. The statistical result revealed that the NTs surface provided a better microenvironment for EC attachment than the Flat Ti, which produced a significant increase of 31.3% in the number of attached cells (Fig. 8B). The PDAM ad-layer on the NTs not only enhanced cell attachment but also stimulated the cell spreading. This may be attributed to the effect of the quinone/phenolic hydroxyl groups of PDAM ad-layer on fetal bovine serum (FBS) adsorption.³⁵ The loading of BVLD to the bare NTs did not produce significant effects on both cell attachment and morphology compared to the control NTs. Loading of BVLD to the PDAM/NTs, resulted in a 34.3% increase in the number of adherent cells. The number of HUVECs adherent on the BVLD-PDAM/NTs was 1.76-fold that on the Flat Ti and 1.34-fold that on the control NTs. Most importantly,

HUVECs attached on the BVLD-PDAM/NTs still showed the spreading state.

With increase in the culture time, the morphology of the cells exhibited significant differences. As reported, the morphology development of ECs is closely related to the expression of functional proteins and mediators, proliferation and migration. Some previous studies have reported that elongated cells are in a state of increased proliferation,³⁸ extracellular matrix production^{19,21} and higher migration speed compared to their spread counterparts.³⁹ The analysis of cell morphology, therefore, might help to gain an insight into the interactions of the cells with substrates. The minor/major ratio (aspect ratio, the value 0 for a straight line and 1 for perfectly round) is an important index of cell morphology, where a lower minor/major ratio of cell indicates a more extended cell. Thus, the projected area of the cells and the aspect ratio of the cultured cells after 1 and 3 days were calculated. As expected, the nanotopography of the NTs showed significant effects on directing cell morphology.^{19,21} The HUVECs grown on the NTs exhibited a smaller projected area (Fig. S7A†) and more elongated morphologies (as evidenced by the smaller aspect ratio of the cells, Fig. S7C†) than HUVECs on the Flat Ti. It was found that the projected area per cell increased but the aspect ratio of the cells decreased with increasing culture time of the HUVECs. This indicates that the cytoskeleton developed along the major axis, and the increased projected area per cell was mainly attributed to the increased length of the cells. Although loading of BVLD to NTs preferred to induce some minor changes in the projected area per cell and the aspect ratio of the cells, the differences were not statistically significant. The modification of the NTs with the PDAM ad-layer led to a significant increase

Fig. 9 (A) Migration of HUVECs on Flat Ti, NTs, PDAM/NTs, BVLD-NTs and BVLD-PDAM/NTs surfaces (the size of sample: 0.8 cm × 2.2 cm, $n = 6$) after 1 day culture. (B) The migration distance and (C) the density of migrated cells; calculated from at least 12 images. Data presented as mean ± SD and analyzed using a one-way ANOVA, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

in the projected area per cell and aspect ratio of the cells. The minor/major axis ratio of the cells on the PDAM/NTs was still smaller than on the Flat Ti. The cells on BVLD-PDAM/NTs had smaller aspect ratios than on PDAM/NTs, although their projected area was almost equal. Elongation of HUVECs was observed at culture on plasma polymerized allylamine coating covalently immobilized with BVLD.³⁴ The cell area coverage on a stent is crucial to the success of the re-endothelialization. The statistical analysis of the cell area coverage revealed that the

PDAM ad-layer played the key role in promoting cell coverage, and the BVLD-PDAM/NTs showed the highest degree of cell area coverage (Fig. S7B†). The statistical analysis of cell morphology indicates that the BVLD-PDAM/NTs surface provides the best microenvironment for cell attachment, spreading, and cytoskeleton development.

To attest the assumption based on the morphology analysis of cells, proliferation, migration and the expression of specific markers of the HUVECs were tested. As shown in Fig. 8C, 9 and 10, the NTs induced a considerable increase in proliferation, migration and NO release of HUVECs as compared to the Flat Ti. The modification by the PDAM ad-layer on the NTs further enhanced cell proliferation, migration and NO release. The loading of BVLD to NTs apparently promoted cell migration better than cell proliferation or NO release. For the BVLD-PDAM/NTs, the combination of dual chemical cues of PDAM ad-layer and BVLD resulted in a substantial enhancement in cell proliferation, migration and NO release. These observations significantly confirmed the analysis of cell morphology.

Migration of normal ECs into a lesion of the vessel wall has been demonstrated to be an important way for the regeneration of endothelium; enhanced EC motility may accelerate wound healing after injury or vascular stent implantation. However, the ECs compete *in vivo* with SMCs. Thus, the efforts to partially promote the EC growth (for instance, based on the EPC capture for rapid re-endothelialization^{14,15}) without considering the *in vivo* competition with SMCs might be misleading and meaningless.^{16,17} Therefore an ideal stent surface should have

Fig. 10 NO levels released in the culture media for Flat Ti, NTs, PDAM/NTs, BVLD-NTs and BVLD-PDAM/NTs (the size of sample: 1.5 cm × 1.5 cm, $n = 4$). Data presented as mean ± SD and analyzed using a one-way ANOVA, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 11 (A) Fluorescence staining of HUVECs (green) and HUASMCs (red) grown onto Flat Ti, NTs, PDAM/NTs, BVLD-NTs and BVLD-PDAM/NTs surfaces (the size of sample: 1 cm × 1 cm, $n = 4$) after culture of 2 h. (B) Number of cells attached on the surfaces is calculated by at least 12 images. (C) The ratio of the number of HUVECs to the number HUASMCs. Data presented as mean ± SD and analyzed using a one-way ANOVA, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

selectivity for ECs and SMCs in a way that *in situ* formation of a healthy endothelium on the stent is induced. Therefore, the test of co-culture of HUVECs and HUASMCs was further carried out. In this work, HUVECs and HUASMCs were seeded in a ratio of 1 : 1 in the competitive adhesion test. Fig. 11 shows the typical fluorescence images of HUVECs (green) and HUASMCs (red) after 2 h culture. On the Flat Ti surface, HUASMCs clearly showed more significant competitive adhesion than HUVECs, the ratio of HUVECs/HUASMCs decreased to 0.49 (Fig. 11C). However, NTs selectively promoted HUVEC adhesion while it inhibited HUASMC adhesion. As shown in Fig. 11B, the number of HUVECs adherence on the NTs was about 1.2-times higher than on the Flat Ti, but the number of HUASMCs on the NTs was only about 59.5% of that on the Flat Ti. The ratio of HUVECs to HUASMCs of the NTs (1.01) was 2.1-times that of the Flat Ti (Fig. 11C). The loading of BVLD to the NTs gave rise to a better HUVEC selectivity and resulted in a higher ratio of HUVECs to HUASMCs (1.24). For the PDAM/NTs, although the number of the adherent HUASMCs increased compared with the NTs, the ratio of HUVECs to HUASMCs (0.93) was close to that of the NTs and was considerably higher than on the Flat Ti. This is attributed to the significant enhancement of PDAM/NTs in HUVEC adhesion. In the case of the BVLD-PDAM/NTs, because of the best promotion in HUVEC adhesion and considerable promotion in HUASMC adhesion, the ratio of HUVECs to HUASMCs reached 1.20 that was considerably higher than the Flat Ti as well as NTs or PDAM/NTs. The excellent hemocompatibility and selective effects of BVLD-PDAM/NTs on HUASMCs and HUVECs suggest the possibility to induce the *in situ* formation of a pure endothelial layer and to address the issues of restenosis and LST.

4. Conclusions

In this work, we have presented a simple surface modification using a poly-dopamine ad-layer to tailor the surface functionalities of TiO₂ nanotube arrays. The TiO₂ nanotube arrays modified by polydopamine ad-layer not only showed a larger capacity of bivalirudin (BVLD) loading than the control nanotube arrays but also exhibited a durable and controllable release of BVLD. The combination of the biological functions of the BVLD and the nanoscale cues of the TiO₂ nanotube arrays provided the multiple functions of improved hemocompatibility and selectivity for endothelial cells (EC) in a competitive growth with vascular smooth muscle cells (VSMCs). These results showed that our material provided an ideal microenvironment that is prone to promote the recovery of endothelium on the luminal surface of a vascular stent and address the long-term complications of restenosis and thrombosis. We believe that this work might have the broad design philosophy of vascular devices and arouse the development of a new generation of vascular stents.

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