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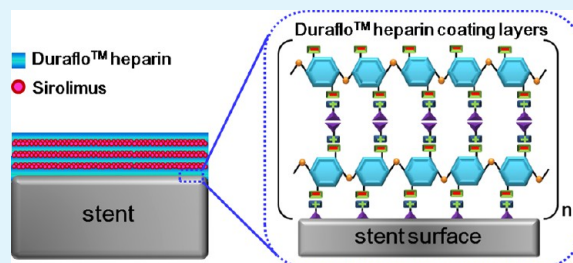
# Dual Drug-Eluting Stents Coated with Multilayers of Hydrophobic Heparin and Sirolimus

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**ABSTRACT:** Polymer coatings for stents are considered one of the key factors that lead to adverse cardiac events after coronary arterial stenting. This study presents a dual drug-eluting stent (DES) that is coated with multilayers of Duraflo heparin and sirolimus but containing no other organic polymers. The hydrophobic Duraflo heparin coating was used to improve the hemocompatibility of the stent and serve as a drug reservoir for the controlled release of sirolimus, thus avoiding inflammatory reactions induced by the conventional polymers. The Duraflo heparin and sirolimus were coated layer-by-layer onto the stent surface using a homemade spray-coating device. The drug loading amount can be easily controlled by adjusting the numbers of layers applied and the concentration of the drug solution, indicating the developed coating process is reproducible and well-controlled. After balloon expansion, the coating did not crack or peel off, which demonstrates that the sirolimus/Duraflo heparin coating layers tightly adhere to the stent surface. The activated partial thromboplastin time (APTT) assay showed that the Duraflo heparin coating significantly prolonged the APTT from  $27.3 \pm 0.3$  s to  $69.7 \pm 6.2$  s, demonstrating the anticoagulant ability of the coated stents. The dual DES exhibited a nearly linear sustained-release profile of Duraflo heparin and an initial burst release followed by a slow release of sirolimus. Less than 15% of heparin was released from the DES within 14 days, indicating the stent can maintain its antithrombotic surface for a long time. Because of the layer-by-layer structure, the most outer layer of Duraflo heparin coating may act as a diffusion barrier to retard sirolimus release from the stent. These results confirm that the dual DESs enable simultaneous delivery of antithrombotic and antiproliferative drugs and have potential for the treatment of coronary artery disease.

**KEYWORDS:** antiproliferation, antithrombosis, drug delivery, layer-by-layer, restenosis, spray coating



## 1. INTRODUCTION

Drug-eluting stent (DES) treatment considerably reduces restenosis compared with bare-metal stent implantation and represents an important advance in percutaneous coronary interventions. First-generation DESs, Cypher (sirolimus-eluting stent; Cordis Corporation, Johnson & Johnson, Warren, NJ) and Taxus (paclitaxel-eluting stent; Boston Scientific Corporation, Natick, Mass) stents, use durable thick polymers as drug reservoirs to store and elute pharmaceutical agents to the lesion site and thus enhance their antirestenotic efficacy. However, the permanent presence of these polymers causes hypersensitivity reactions and chronic inflammation responses in the vessel wall.<sup>1–4</sup> To overcome these unfavorable effects, new DES platforms have focused on using more biocompatible and biodegradable polymers as alternatives. Stents that seem to be especially appealing to medical professionals are those covered with a biodegradable polymer coating that carries and controls the drug release during a proper period of time, then erodes and disappears from the artery.<sup>5</sup>

Several biodegradable polymers have been evaluated in different experimental and preclinical animal models with respect to their biocompatibility for in vivo use.<sup>6–8</sup> The most commonly used polymers now are poly(lactic acid) (PLA);

poly(glycolic acid) (PGA); and copolymer, poly(lactic-co-glycolic acid) (PLGA) because they can degrade through hydrolysis into metabolites that can be safely eliminated from the body through citric acid cycles.<sup>9</sup> Although DES with biodegradable coatings considerably improve overall clinical outcomes and reduce stent thrombosis compared with DES that are coated with a durable polymer,<sup>10–13</sup> the acidic products generated from polymer degradation (i.e., lactic acid and glycolic acid) still elicit various degrees of inflammatory responses on the vessel wall.<sup>8,13,14</sup> Rapid degradation of polymeric coatings usually causes a burst release of acidic products that may result in an uncontrollable inflammatory reaction.<sup>8</sup> Therefore, minimizing the arterial inflammation caused by the polymer coating represents a challenge.

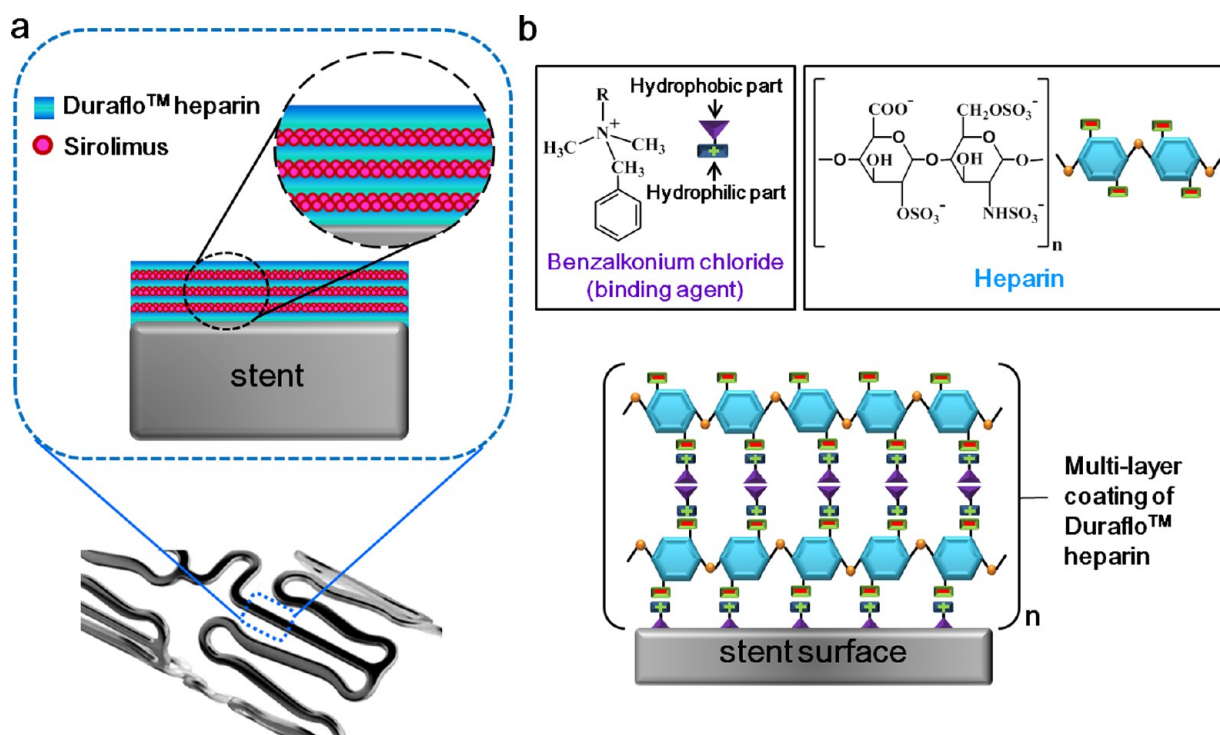
This study presents a dual DES that possesses unique Duraflo heparin coating layers to replace traditional polymer coating for carrying antiproliferative drugs, sirolimus (Figure 1). The DES was prepared by spraying Duraflo heparin and sirolimus solutions layer-by-layer onto the surface of a metallic

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**Figure 1.** Schematic illustrations of the dual drug-eluting stent. (a) Multilayer coatings of Duraflo heparin and sirolimus on the stent surface. (b) The coating composition of the Duraflo heparin. The Duraflo heparin coating is accomplished by attaching heparin to the stent surface through a proprietary binding agent, benzalkonium chloride.

stent using a homemade device. Duraflo heparin, a water-insoluble heparin-quaternary ammonium complex, is composed of unfractionated heparin and a proprietary hydrophobic binding agent, benzalkonium chloride (Figure 1b).<sup>15</sup> Duraflo heparin coating is accomplished by attaching heparin to the stent surface through the binding agent that has high affinity to a variety of synthetic surfaces. Duraflo heparin treatment has been revealed to improve the blood compatibility of cardiopulmonary-type devices by preventing the activation of the blood-clotting system that naturally occurs when blood comes in contact with foreign materials.<sup>16,17</sup> Previous studies have reported that the ionically coated heparin complex can be coated onto a stent in multiple layers and can exert its antithrombotic effects for a longer time compared to a stent with a monolayer of covalently bound heparin.<sup>18,19</sup>

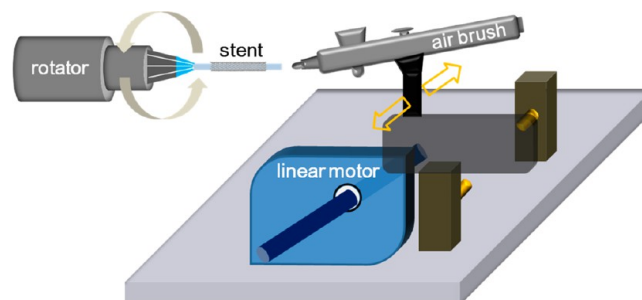
Sirolimus, a natural macrocyclic lactone, is a potent immunosuppressive agent approved by the Food and Drug Administration. It has been proven to inhibit the growth of vascular smooth muscle cells, thus reducing neointimal hyperplasia.<sup>20,21</sup> A combination of antithrombotic and anti-proliferative therapy would be a practical and effective approach to prevent thromboischemic events and neointima hyperplasia.<sup>22</sup> The aim of this study was to develop a new DES that allows dual drug release of sirolimus and heparin to inhibit both restenosis and thrombosis without recourse to a carrier polymer. In this study, preparation of the dual DES, composed of multilayers of Duraflo heparin and sirolimus, was reported. Additionally, its *in vitro* characteristics and drug release profiles were investigated.

## 2. EXPERIMENTAL SECTION

**2.1. Materials.** Duraflo heparin (MW = 49–66 kDa) and sirolimus (Rapamycin) were obtained from Edwards Life Science (Irvine, CA, USA) and LC Laboratories (Woburn, MA, USA), respectively. The

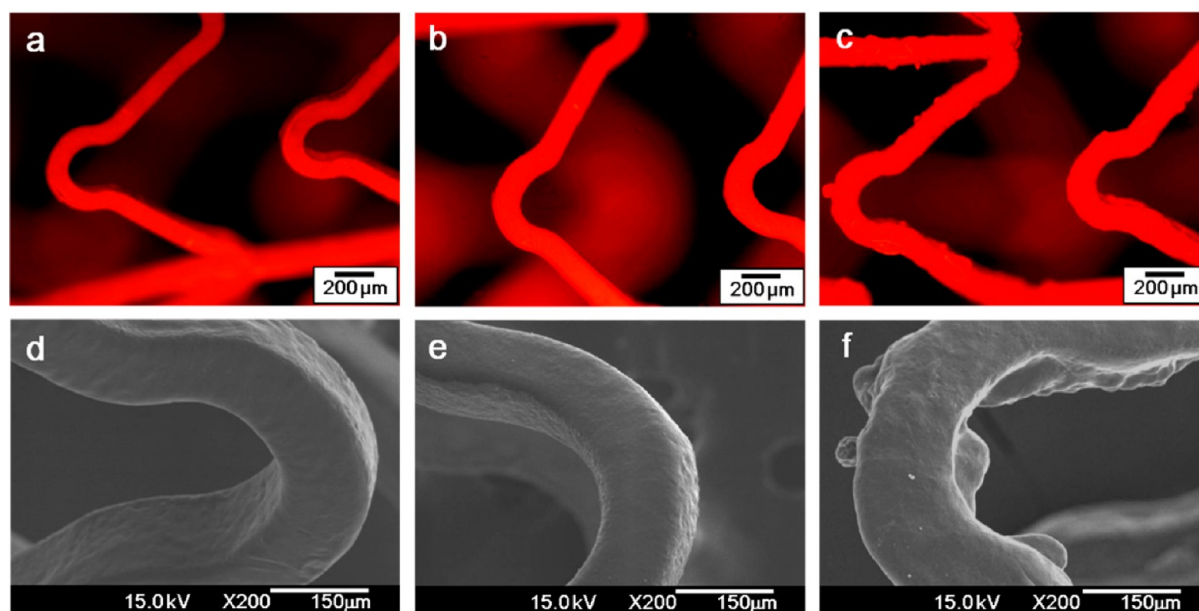
metallic stents used in the study were the Multi-Link PIXEL stent (Guidant, Santa Clara, CA, USA), which is a 316 L stainless-steel stent with a length of 13 mm. Rhodamine 6G, hexane, isopropyl alcohol, toluidine blue o, phosphate buffered saline (PBS), methanol, and ethanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). All chemicals and reagents used were of analytical grade.

**2.2. Preparation of Duraflo Heparin-Coated Stents.** To select an optimal concentration for spray coating, test solutions (20, 30, and 40 mg/mL) were prepared by dissolving Duraflo heparin in a mixture of 78% hexane and 22% isopropyl alcohol (by volume). The prepared Duraflo heparin solution was loaded in an air brush with a 0.3-mm nozzle (Rich AS-3, Japan) that was controlled by a linear motor (US-51, Fu Chang Co., Taoyuan, Taiwan, Figure 2). The stent was



**Figure 2.** Schematic illustrations of the homemade spray-coating equipment, including an air brush, a rotator, and a linear motor. The stent is mounted on a mandrill that is driven by the rotator.

mounted on a polytetrafluoroethylene mandrill that was driven by a rotator. The loaded solution in the air brush was then spray-coated layer-by-layer on the abluminal surface of the mounted stent (0.25 mL of Duraflo heparin solution per layer). Each coating layer must be allowed to dry before the next layer is applied. Rhodamine 6G (a fluorescent red dye, 400 ppm) was added to the solution to determine whether Duraflo heparin was successfully coated on the stent surface.



**Figure 3.** (a–c) Fluorescence microscopic images and (d–f) SEM micrographs of stents coated with (a, d) 20, (b, e) 30, and (c, f) 40 mg/mL of Duraflon heparin solution.

The Duraflon heparin-coated stent was allowed to dry in air for 2 h and subsequently examined by a fluorescence microscope (IX-71, Olympus, Tokyo, Japan) and a scanning electron microscope (SEM; S4000, Hitachi, Tokyo, Japan).

**2.3. Determination of Heparin Content on the Stent.** To determine the relationship between the number of sprayed layers and the amount of heparin coated onto the stent, stents were spray-coated with 1, 2, 3, 4, or 5 layers of Duraflon heparin solution (30 mg/mL, 0.25 mL per layer). The heparin content coated on the stents was assayed by a toluidine-blue colorimetric method, which was slightly modified from a published method.<sup>23</sup> In brief, the test stent was immersed in 1 mL of ethanol and sonicated for 1 h to dissolve the coated Duraflon heparin. The sample solution was then mixed with 1 mL of 0.2% (w/v) NaCl aqueous solution and dried in the oven at 37 °C overnight. The residue was redissolved in 1 mL of deionized (DI) water and then well mixed with 1 mL of 0.0025% (w/v) toluidine blue solution containing 0.2% (w/v) NaCl and 0.01 N HCl for 30 s. The absorbance at 631 nm was determined for each sample by using a multimode microplate reader (Infinite M200, Tecan Group Ltd., Männedorf, Switzerland).

**2.4. Determination of Coating Thickness.** To measure the thickness of the coating layer on the stent, we carefully applied a scratch to the coating using a needle, all the way to the surface of the metallic stent. After being scratched, the stent surface was then observed by SEM.

**2.5. Balloon-Expansion Test.** The test stent was mounted on a commercial angioplasty balloon (Guidant, Temecula, CA, USA) and the balloon was inflated to a maximum pressure of 10 atm for 30 s, then deflated and slowly withdrawn. Subsequently, the coatings on the stent were examined by SEM.<sup>24</sup>

**2.6. Activated Partial Thromboplastin Time (APTT) Measurement.** The anticoagulant property of Duraflon heparin-coated stent has been evaluated by an APTT assay. APTT was used to detect the intrinsic coagulation, i.e., the influence on the coagulation factors.<sup>25</sup> Briefly, fresh anticoagulant whole blood of a volunteer was first centrifuged at 3000 rpm for 10 min to obtain platelet-poor plasma (PPP). Samples were immersed in 1 mL of PPP and incubated at 37 °C for 30 min. Subsequently, 100 μL of incubated PPP was transferred to a test tube and 100 μL of APTT reagent was added to the same test tube and incubated at 37 °C for 3 min. Finally, 100 μL of an aqueous 0.025 M CaCl<sub>2</sub> solution was added and mixed well. The coagulation time of each sample was measured on an automatic coagulation

analyzer (Sysmex CA-1500, Kobe Japan). The data were averaged from measurements on four specimens.

**2.7. Preparation of Dual DES.** A similar spray coating method as described in Section 2.2 was used to fabricate dual DESs. To investigate the effect of sirolimus concentration on the drug loading dose, Duraflon heparin solution (30 mg/mL, 0.25 mL per layer) and sirolimus (0.5, 1, 2, 3, or 5 mg/mL in ethanol, 0.25 mL per layer) were sprayed layer-by-layer onto the stent surface. In this group, all DESs were coated with 3 layers of Duraflon heparin and 2 layers of sirolimus.

To explore the effect of the number of sprayed layers on the loading dose, 30 mg/mL of Duraflon heparin solution (0.25 mL per layer) and 3 mg/mL of sirolimus solution (0.25 mL per layer) were used for the spray coating. Three types of stents with different numbers of coating layers were prepared. For the low-dose stent, two layers of Duraflon heparin and one layer of sirolimus were spray-coated on the stent. For the medium-dose stent, three layers of Duraflon heparin and two layers of sirolimus were sprayed onto the stent. For the high-dose stent, there were four layers of Duraflon heparin and three layers of sirolimus.

**2.8. Determination of Sirolimus Content on the Stent.** To extract sirolimus from the DES, the test stent was immersed in ethanol and sonicated for 1 h. The extracts obtained were filtered through a 0.22 μm syringe filter and then analyzed by high-performance liquid chromatography (HPLC) equipped with a C18 reversed-phase column (4.6 × 250 mm, particle size 5 μm, ThermoQuest, BDS, Runcorn, UK). The flow rate of the mobile phase (85% methanol, 15% DI water, and 0.1% acetic acid by v/v/v), delivered by an HPLC pump (Series 1500, LabAlliance, State College, PA, USA), was 1 mL/min at 50 ± 1 °C. The eluent was monitored with a UV detector (Model 201, LabAlliance, State College, PA, USA) at 254 nm.<sup>20</sup>

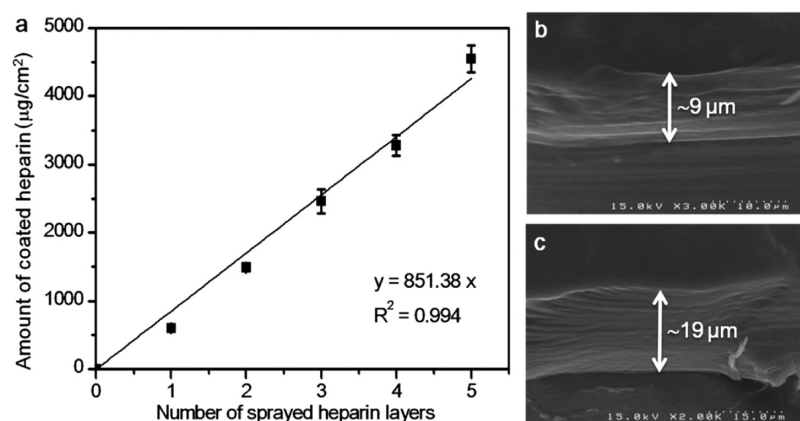
The sirolimus loading efficiency (LE) and loading content (LC) of DESs were calculated using the following equations.

$$LE(\%) = \frac{\text{weight of sirolimus in the coating layer}}{\text{weight of the sprayed sirolimus}} \times 100\%$$

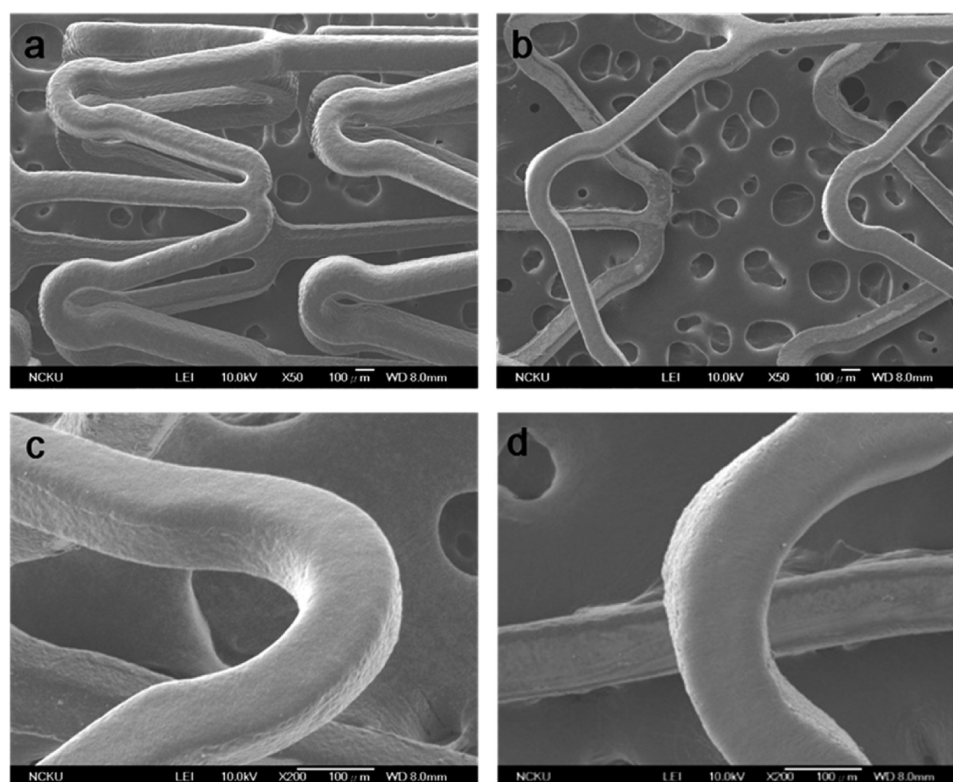
$$LC(\%) = \left( \frac{\text{weight of sirolimus in the coating layer}}{\text{weight of the coating layer (including Duraflon heparin and sirolimus)}} \right) \times 100\%$$

**2.9. In Vitro Drug Release Study.** The prepared DESs were well immersed into the release medium, which was composed of sterilized PBS (pH 7.4) or PBS containing 10% (v/v) or 20% (v/v) ethanol, and





**Figure 4.** (a) Relationship between the number of sprayed heparin layers and the amount of heparin coated onto the stent ( $n = 3$ ). SEM micrographs of the cross-sectional morphologies of stents coated with (b) 2 and (c) 4 layers of DuraFlo heparin. The double-headed arrows indicate the thickness of the DuraFlo heparin coating.



**Figure 5.** SEM micrographs of DuraFlo heparin-coated stent (a, c) before and (b, d) after balloon expansion. (c, d) show higher magnifications of a and b, respectively.

contained in test tubes.<sup>26</sup> The tubes were then incubated in a water-bath shaker at 37 °C at 120 rpm. The release medium in each tube was collected and replaced with a fresh one at specified times. The collected sample was then well mixed with an equivalent volume of ethanol to fully dissolve the released drugs. Concentrations of sirolimus and DuraFlo heparin in the medium were determined by HPLC and a toluidine blue colorimetric method, respectively.

**2.10. Statistical Analysis.** For this study, we compared the two groups using the one-tailed student  $t$  test by employing statistical software (SPSS, Chicago, IL, USA). This study presents data as the mean  $\pm$  SD. A difference of  $p < 0.05$  was considered statistically significant.

### 3. RESULTS

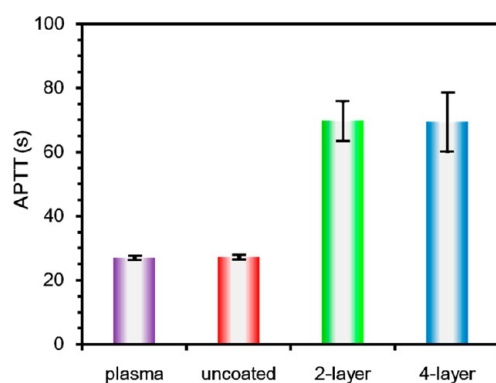
**3.1. Characterization of the DuraFlo Heparin-Coated Stent.** Solution concentration is one of the major parameters that affect the performance of spray coating. To determine the optimal condition for a smooth and uniform coating of DuraFlo heparin, we tested solutions with concentrations of 20, 30, and 40 mg/mL. Test solutions were prepared by dissolving DuraFlo heparin in a mixture of 78% hexane and 22% isopropyl alcohol (by volume). The DuraFlo heparin coatings on the stents can be adequately visualized by the use of Rhodamine 6 G, a red fluorescence. Uniformity and coverage of coating were checked by fluorescence microscopy and SEM (Figure 3). We discovered that at higher solute concentrations (40 mg/mL),

the solvent evaporation is too fast. Spreading such droplets over the stent surface becomes difficult and forms a rough and uneven coating layer (Figure 3c, f). By contrast, the use of lower concentrations (20 and 30 mg/mL) allows a more desirable droplet spreading, which leads to a smoother and more uniform coat (Figure 3a, b, d, e). In addition, the fluorescence intensity of a stent coated with 20 mg/mL of Duraflo heparin (Figure 3a) was weaker than that coated with 30 mg/mL (Figure 3b), indicating a less amount of heparin was coated onto the stent surface. To achieve a higher coating efficiency and superior coating uniformity, we used a Duraflo heparin solution of 30 mg/mL (viscosity  $\sim 0.78$  mPa·s at 25 °C) for the remainder of the study.

To evaluate the effect of the number of sprayed layers on the amount and thickness of coated heparin, stents were spray coated with 1, 2, 3, 4, or 5 layers of Duraflo heparin (0.25 mL per layer). Figure 4a shows the relationship between the number of sprayed heparin layers and the amount of heparin coated onto the stent. As shown, with the increase in the number of sprayed layers, the amount of the heparin coated on the stent increased and exhibited a linear relationship ( $R^2 = 0.994$ ,  $n = 3$ ). Figure 4b,c shows the representative SEM micrographs of the cross-sectional morphologies of stents coated with two and four layers of Duraflo heparin. As shown, the four-layer coating (ca. 19  $\mu\text{m}$ ) is approximately twice as thick as the two-layer coating (ca. 9  $\mu\text{m}$ ), which indicates that the thickness of the coating layer depends on the number of coatings applied. These results indicate that the developed spray coating process is well-controlled and that the heparin content on the stent surface and the coating thickness can be accurately predicted.

The morphologies of Duraflo heparin-coated stents, before and after expansion, were investigated by SEM. Figure 5 shows the Duraflo heparin coating remained intact without cracking or delamination even after balloon expansion, indicating that the coating possess enough adhesion and mechanical stability to allow the stent to expand.

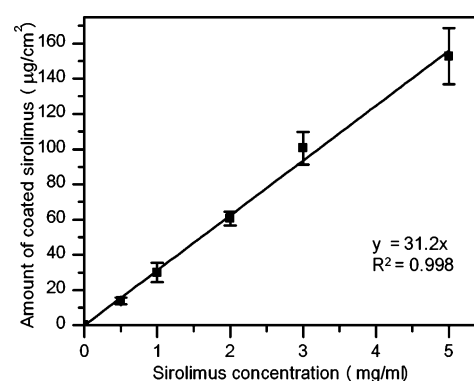
**3.2. Anticoagulant Property of the Duraflo Heparin-Coated Stent.** The APTT test was conducted to evaluate the anticoagulant activity of the samples. We found the APTT values of all Duraflo heparin-coated stents were significantly prolonged compared with the control plasma and the uncoated stents ( $p < 0.05$ , Figure 6). This result demonstrated that the Duraflo heparin coating improves the blood compatibility of



**Figure 6.** Activated partial thromboplastin time (APTT) of stents coated with 2 and 4 layers of Duraflo heparin. The poor-platelet plasma and uncoated stents were used as references. ( $n = 4$  for each group).

the stent. The anticoagulation property of stent was affected by the amount of heparin that was exposed on the stent surface. Therefore, no significant difference between the stent coated with 2 or 4 layers of heparin was found ( $p > 0.05$ , Figure 6).

**3.3. Drug Loading Amount of the Dual DES.** In this study, dual DESs were fabricated by spraying Duraflo heparin and sirolimus layer-by-layer onto the surface of the stent. To explore the effect of sirolimus concentration on the drug loading dose, we used sirolimus solution with a concentration of 0.5, 1, 2, 3, or 5 mg/mL for spray coating. The prepared DESs consist of 3 layers of Duraflo heparin and 2 layers of sirolimus (0.25 mL per layer). We demonstrated that the amount of the sirolimus coated on the stent was linearly related to the drug concentration ( $R^2 = 0.998$ ,  $n = 3$ , Figure 7). Table 1



**Figure 7.** Relationship between the concentration of sirolimus solution and the amount of sirolimus coated onto the stent ( $n = 3$  for each group).

**Table 1. Sirolimus Loading Dose (loading amount/stent surface area), Loading Efficiency (LE), and Loading Content (LC) of Drug-Eluting Stents Coated with Various Concentrations of Sirolimus Solution ( $n = 3$ )**

conc (%)	dose ( $\mu\text{g}/\text{cm}^2$ )	LE (%)	LC (%)
0.5	13.8 $\pm$ 2.0	5.5 $\pm$ 0.8	0.6 $\pm$ 0.1
1.0	29.9 $\pm$ 5.4	6.0 $\pm$ 1.1	1.2 $\pm$ 0.2
2.0	60.6 $\pm$ 3.9	6.1 $\pm$ 0.4	2.4 $\pm$ 0.2
3.0	100.5 $\pm$ 9.4	6.7 $\pm$ 0.6	4.1 $\pm$ 0.4
5.0	152.7 $\pm$ 15.9	6.1 $\pm$ 0.6	6.2 $\pm$ 0.6

shows the sirolimus loading dose (loading amount/stent surface area), as well as the LE and LC of DESs coated with various concentrations of sirolimus solution. As expected, the loading dose and the LC increased with an increase in the drug concentration.

To fabricate low-dose, medium-dose, and high-dose DESs, we spray-coated stents with different layers (0.25 mL per layer) of Duraflo heparin solution (30 mg/mL) and sirolimus solution (3 mg/mL). Table 2 shows the loading doses of sirolimus and Duraflo heparin on the prepared DESs. As shown, the amount of the drug coated on the stent was approximately proportional to the number of its coating layers ( $R^2 = 0.999$  for sirolimus;  $R^2 = 0.994$  for Duraflo heparin). These results showed that the sirolimus loading amount can be easily controlled by adjusting the concentration of the drug solution and the number of coating layers.

**3.4. Characterization of the Dual DES.** The fabricated dual DESs were expanded by a balloon-catheter and their surface morphologies were monitored by SEM before and after

**Table 2. Sirolimus and Duraflo Heparin Loading Doses (loading amount/stent surface area) of Drug-Eluting Stents (DESs) Coated with Different Numbers of Sirolimus and Duraflo Heparin Layers ( $n = 3$ )**

DESs	coating layers (sirolimus/heparin)	sirolimus dose ( $\mu\text{g}/\text{cm}^2$ )	heparin dose ( $\mu\text{g}/\text{cm}^2$ )
low-dose	1/2	$51.1 \pm 10.1$	$1490 \pm 20$
medium-dose	2/3	$100.5 \pm 9.4$	$2462 \pm 175$
high-dose	3/4	$147.7 \pm 17.5$	$3281 \pm 151$

expansion to verify the coating integrity (Figure 8). As shown, the sirolimus/Duraflo heparin coating on stent was uniform and smooth. The stent expansion did not induce fissures and the cohesion of the sirolimus/Duraflo heparin coating was maintained, indicating that incorporation of sirolimus between Duraflo heparin layers does not influence the adhesion between the coating layers and stent surface. Figure 9 shows the representative SEM images of a low-dose and a high-dose DESs after being cut with a knife. The thickness of sirolimus/Duraflo heparin coating was measured by SEM image analysis.<sup>27</sup> When compared with Figure 4b and 4c, we found a slight increase in the coating thickness due to incorporation of sirolimus into the coating layers.

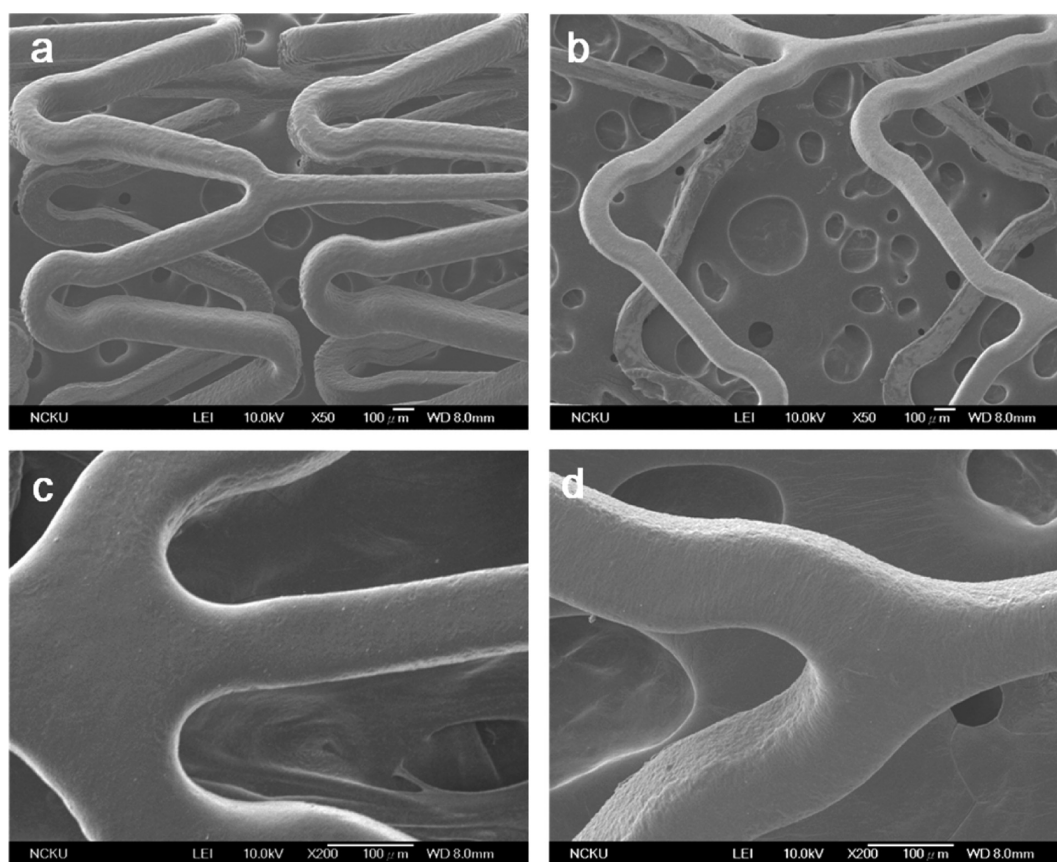
**3.5. In Vitro Release Profiles of Sirolimus and Duraflo Heparin.** Sirolimus and Duraflo heparin are practically insoluble in water, but soluble or slightly soluble in ethanol.<sup>28</sup> Because of these solubility considerations, PBS containing 10% or 20% (v/v) ethanol was used as a release medium for the in

vitro drug release study.<sup>26,29</sup> Figure 10 displays the accumulative release profiles of Duraflo heparin and sirolimus from the low-dose stent at different ethanol concentrations. As shown, significant increases in the drug release in the ethanol-containing buffers were observed compared to the no ethanol condition (PBS only) ( $p < 0.05$ ).

As shown in Figure 10a, no release of Duraflo heparin was detected in the PBS medium, but a near zero-order release was observed in the presence of ethanol. The release rate of Duraflo heparin was dependent on ethanol concentration and a higher release percentage was reached in the 20% ethanol medium (18%) compared to the 10% ethanol medium (11%) on day 10, thus revealing that Duraflo heparin can be gradually released from the stent surface in the ethanol-containing medium.

Compared to the Duraflo heparin, sirolimus is slightly soluble in water with a solubility of  $2.6 \mu\text{g}/\text{mL}$ .<sup>28</sup> Figure 10b shows that a little amount of sirolimus was detected in the pure PBS. For the ethanol-containing medium, the release of sirolimus from the stent exhibited an initial burst release within the first day, followed by a slower continued release until a plateau was reached. On day 10, a cumulative sirolimus release of 25 and 42% was observed for ethanol concentrations of 10 and 20%, respectively, compared to that of 5% observed with PBS.

In this study, the release behavior of sirolimus from DESs in the ethanol-containing medium can be explained by a certain set of phenomena. The low-dose stent was coated with 2 layers of Duraflo heparin and 1 layer of sirolimus (Figure 11). Each layer of heparin contains approximately 50% of total heparin



**Figure 8.** SEM micrographs of dual DESs (a, c) before and (b, d) after balloon expansion. c and d show higher magnifications of a and b, respectively.



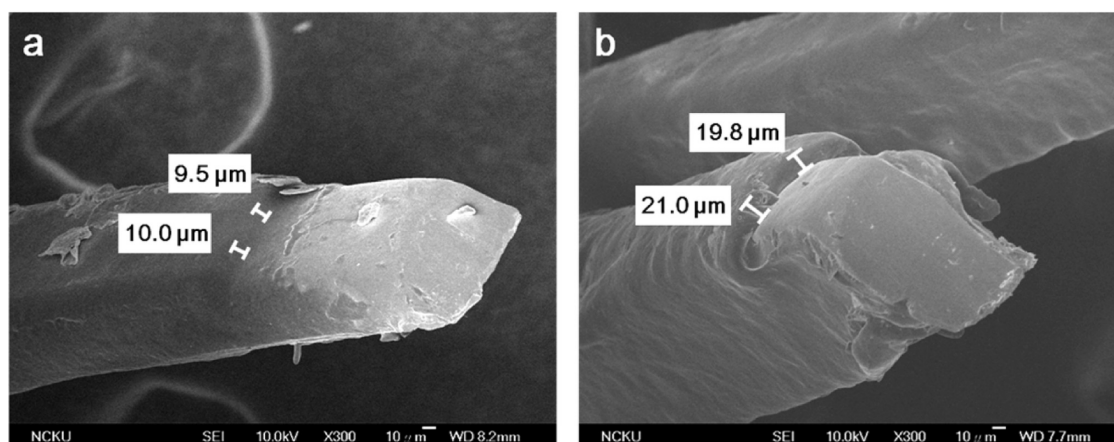


Figure 9. SEM micrographs of (a) low-dose and (b) high-dose DESs after being cut with a knife.

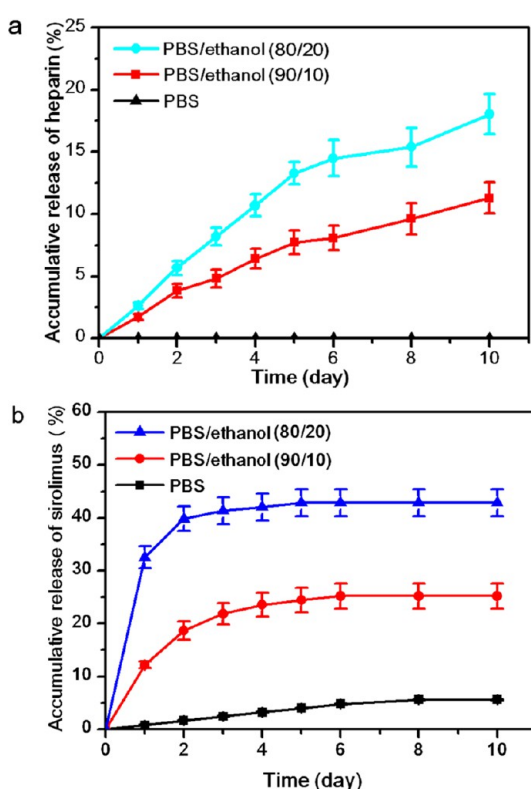


Figure 10. Accumulative release profiles of (a) DuraFlo heparin and (b) sirolimus from the low-dose stent at different ethanol concentrations: pure PBS and PBS containing 10% or 20% (v/v) ethanol ( $n = 3$  for each group).

loading. The burst release of sirolimus may be caused by the dissolution of the drug located at or close to the surface of the coating and this effect is likely dependent on ethanol concentration. The slow release may be caused by the diffusion of sirolimus from the DuraFlo heparin coating and is likely affected by the DuraFlo heparin's dissolution rate. After 10 days of release, less than 20% of heparin was released from the outer heparin coating (Figure 10a), suggesting that most sirolimus was located between the inner and outer layers of heparin (Figure 11). The remaining DuraFlo heparin of the outer layer may act as a diffusion barrier, thus retarding sirolimus release from the stent.

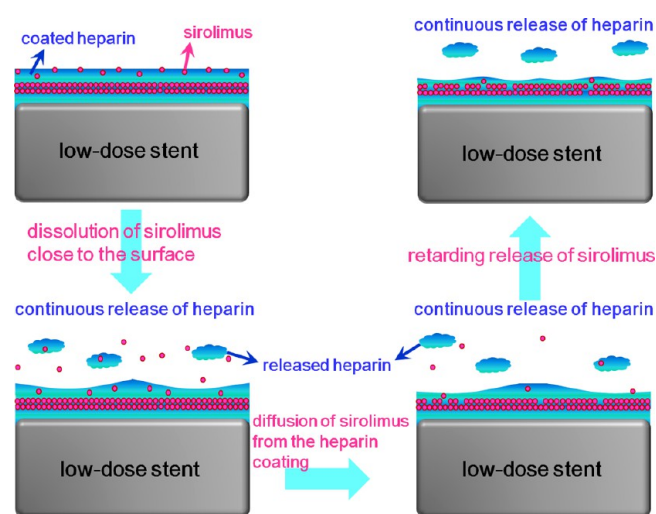
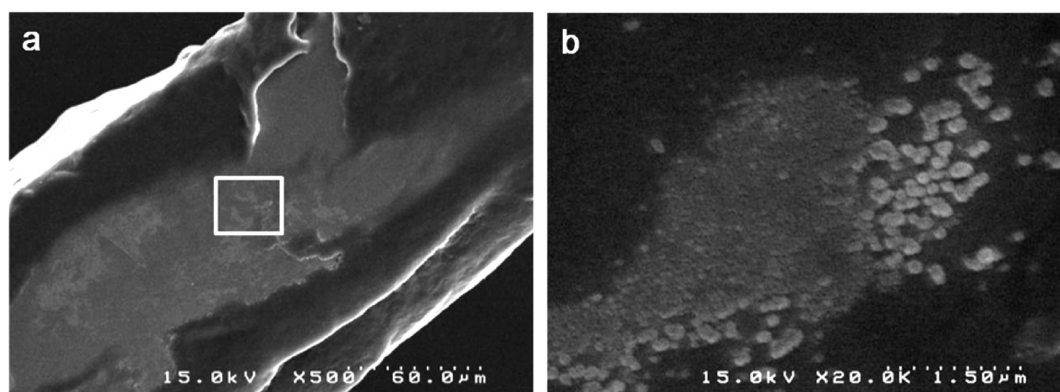


Figure 11. Schematic representation of a hypothetical release behavior of sirolimus and DuraFlo heparin released from the low-dose stent in the ethanol-containing buffer.

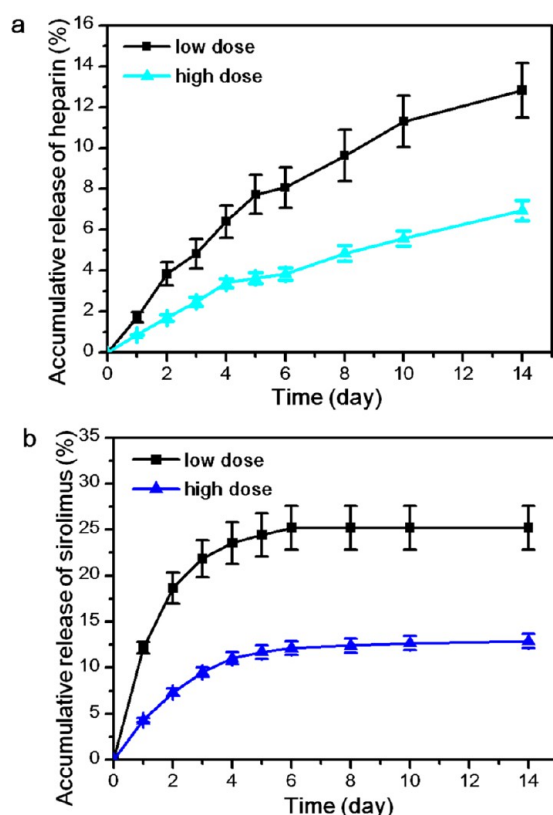
Because DuraFlo heparin is slightly soluble in the ethanol-containing buffer, a slower and continuous release of DuraFlo heparin can be observed (Figures 10a and 11). Figure 12 shows the sample surface morphologies characterized by SEM after the drug release study. As shown, heparin release from the stent resulted in partial disappearance of the outermost coating (Figure 12a). Some drug crystals were observed at that region (Figure 12b), indicating that a small part of sirolimus layer, originally located in the middle layer, was exposed to the stent surface. These results provided an evidence of the layer-by-layer structure of DuraFlo heparin and sirolimus coating.

Figure 13 shows the accumulative release profiles of DuraFlo heparin and sirolimus from the low-dose and the high-dose stent in the PBS containing 10% ethanol. As shown, these two DESs exhibited similar drug release behaviors. The high-dose stent exhibited a lower release percentage compared to that of the low-dose stent, which may provide a more prolonged drug release. After 14 days of release, approximately 12.8 and 6.9% of heparin and 25.2 and 12.9% of sirolimus were released from the low-dose and high-dose stent, respectively.





**Figure 12.** SEM micrographs of low-dose DES after 10 days of release in the ethanol-containing buffer. (b) High-magnification image of the white square in a.



**Figure 13.** Accumulative release profiles of (a) Duraflo heparin and (b) sirolimus from the low-dose and the high-dose stents in the PBS containing 10% (v/v) ethanol ( $n = 3$  for each group).

#### 4. DISCUSSION

In-stent thrombosis remains an important concern after stent implantation. Stents are foreign bodies in the vessel wall and thus induce platelet adhesion, activation, and thrombus formation. Most stent thrombosis occurred within the first 10 days of the procedure; after the first month these events were extremely rare when bare metal stents were used.<sup>30,31</sup> Despite considerably reduced restenosis rates, DES carries a high risk of late stent thrombosis.<sup>32–34</sup> Drug release from DES is intended to inhibit vascular smooth muscle cell proliferation and migration, but it also impairs reendothelialization.<sup>3</sup> Incomplete endothelial coverage and neointimal coverage over strut after DES implantation could be a possible cause of late thrombosis. In addition, polymers used to encapsulate these antiproliferative

drugs have been associated with DES thrombosis.<sup>3</sup> Some stent thrombosis may lead to mortality or acute myocardial infarction.<sup>35</sup> Therefore, avoiding thrombus formation after stent implantation is critical.

This study presents a heparin- and sirolimus-eluting stent to simultaneously provide antithrombotic and antiproliferative therapy for the treatment of coronary stenosis. To reduce the deleterious effects induced by the conventional polymer and improve the hemocompatibility of the stent, we employed the hydrophobic Duraflo heparin coating as a drug reservoir to encapsulate the antiproliferative agent. The dual DES, composed of multilayers of Duraflo heparin and sirolimus (Figure 1), was prepared by using a homemade spray-coating device (Figure 2). The drug loading amount can be easily controlled by adjusting the number of layers applied (Figure 4) or the concentration of the drug solution (Figure 7).

Duraflo heparin is a hydrophobic heparin-quaternary ammonium complex, formed by ionic bonding of the anionic heparin to the cationic binding agent, benzalkonium chloride (Figure 1b).<sup>15,36,37</sup> Benzalkonium chloride, a positively charged surfactant, is commonly used to prevent bacterial contamination for medical disinfection.<sup>38</sup> In this study, the APTT test showed that Duraflo heparin coating significantly improves the blood compatibility of the metallic stent (Figure 6). The APTT is a standard test of the intrinsic coagulation and is widely used to monitor unfractionated heparin therapy.<sup>39</sup> Our results demonstrated that the binding agent is incorporated with heparin in such a way that only affects the physicochemical but not the biological properties of heparin. A previous study also reported that the Duraflo heparin coating exerts excellent antithrombotic activity to inhibit thrombus formation in porcine coronary arteries.<sup>18</sup>

Balloon expansion test showed that no cracking or peeling of the coating layers was observed after stent expansion (Figures 5 and 8). These results demonstrated that Duraflo heparin coating has strong adhesion to the stent surface, which may be attributed to the hydrophobic interaction between the hydrocarbon tail of the binding agent and the hydrophobic stent surface. During stent expansion, traditional polymer coating may crack and release polymer fragments, which may result in an inflammatory or acute thrombosis.

Stent coatings are designed to improve stent biocompatibility and deliver drugs to enhance its performance. Coating materials play a vital role in tissue repair and remodeling. Once the drug has been eluted, the residual polymer coating, which is either durable or absorbable, is believed to be associated with

impaired vascular healing and chronic inflammation, or extremely late stent thrombosis.<sup>13,40</sup> Using this dual DES system may reduce the incidence of untoward side effects because no polymer remains on the stent after drugs have been released.

The in vitro drug release study revealed that coated Duraflo heparin can be slowly and continuously released from the stent surface. Duraflo heparin release from the stent surface may be dominated by “surface erosion”. The erosion rate is directly proportional to the surface area of the heparin coating. For a very thin matrix, the surface area remains relatively constant when the matrix erodes, which allows surface erosion (or drug release) to be characterized as zero order release.<sup>41</sup> As shown in Figure 13a, more than 85% of the heparin still remained on the stent after 14 days of release, indicating that the developed DES can maintain its antithrombotic surface for a long time. Due to the layer-by-layer structure of coating, the most outer layer of Duraflo heparin coating serves as a diffusion barrier to achieve an extended release of the most encapsulated sirolimus (Figures 10b, 11, and 13b). We hypothesize that after the antiproliferative drugs were completely released, the most inner heparin coating still provided a protection against thrombosis.

Ideally, the in vitro release conditions should mimic the physiological environments<sup>42,43</sup> in which body fluid is constantly being exchanged. For drugs with low solubility in the release medium, a small amount of surfactants or solvents would be added to the release buffer to maintain an infinite sink condition.<sup>26,29</sup> The infinite sink condition means the concentration of a drug in the release medium does not exceed 10% of its solubility.<sup>44</sup> It has been reported that sirolimus solubility in ethanol was higher than 90 mg/mL.<sup>28</sup> Although the recommended solvent for Duraflo heparin is a mixture of 78% hexane and 22% isopropyl alcohol, we found this solvent is immiscible with PBS because of its low polarity. To find an alternative, we tried to dissolve Duraflo heparin in ethanol and found it can be dissolved in ethanol with a solubility of at least 5 mg/mL. To increase drug solubility in the release medium, ethanol was added in the PBS medium for the drug release study. However, it should be noted that the blood composition is very complex and the in vitro assays might not truly or exactly mimic in vivo conditions for drug release.

## 5. CONCLUSION

We developed a dual DES that uses a unique hydrophobic heparin to carry antiproliferative drugs such as sirolimus. This study demonstrates that the coated heparin and sirolimus can be gradually released from the stent surface, which may exert antithrombotic and antiproliferative effects after stent implantation. We suggested that the developed dual DES has the potential to avoid any polymer-induced adverse effect and may serve as an alternative for treatment of coronary artery stenosis.

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### Notes

The authors declare no competing financial interest.

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