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Drug loaded nanoparticle coating on totally bioresorbable PLLA stents to prevent in-stent restenosis

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Abstract: Biodegradable polymer poly (DL-lactide) (PDLLA) has been used as drug coating material for drug-eluting stents due to its excellent biocompatibility and sustained drug release ability. However, the uniform thin layer drug eluting coating on a stent not only inhibits the blood vessel's smooth muscle cell overgrowth but also delay the endothelialization process which is often associated with the occurrence of acute thrombosis. Therefore, in this study, we developed a novel coating method using PDLLA nanoparticles (NPs) as a coating to overcome this issue. The average 300 nm sized sirolimus-loaded PDLLA nanoparticles were prepared by a conventional emulsion solvent evaporation method. A low temperature plasma polymerization technology to graft hydrophilic polymers on to poly (L-lactide) stent was used to increase the surface coating efficiency of nanoparticles on the stent. Results showed that sirolimus-loaded nanoparticles can be successfully coated on to the stents with sustained drug release properties. *In vitro* cell culture study showed the drug loaded nanoparticle coating effectively inhibited the proliferation of smooth muscle cells while still allowed a faster proliferation of endothelial cells, suggesting that the new NP coated bioresorbable stents have the potential to reduce both the occurrence of in-stent restenosis and acute thrombosis. © 2016 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 00B: 000–000, 2016.

Key Words: bioresorbable stents, poly (lactide) nanoparticle, stenosis, plasma polymerization, human coronary artery smooth muscle cells (HCASMCs), human coronary artery endothelial cells (HCAECs)

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INTRODUCTION

The first angioplasty was performed by doctor **G**ruentzig in 1977, which is the start of the new epoch of percutaneous transluminal coronary angioplasty (PTCA). After that, this field evolved rapidly and has gone through PTCA, bare metal stent (BMS) and drug-eluting stent (DES) eras.[1] Recently, drug-eluting stents (DES) loaded with anti-proliferative drugs, such as sirolimus or paclitaxel, have been used extensively in percutaneous coronary interventions for the prevention of restenosis.[2] Clinical application indicated that DES is successful in dealing with the acute vascular occlusion after PTCA and the postoperative restenosis of BMS.[3–5] But DES has its own issues: these permanently existed metal implants in vessels will limit the normal movement of blood vessels and cause inflammatory reaction of vascular wall, which in turn will lead to a sequence of immunological and biochemical reactions as well as arterial wall hyperplasia and in-stent restenosis. Moreover, the antiproliferative drug coating on the surface of DES suppresses the vascular smooth muscle hyperplasia while delay the renovation of vascular endothelium and result in late in stent thrombosis.[6–8]

To overcome these obstacles that conventional DES have faced, totally bioresorbable stents were developed. These types of stents can be completely resorbed *in vivo*, thereby eliminating the potential complications of arterial rupture, tissue intimal hyperplasia, and late in stent thrombosis. They will also reduce neointimal hyperplasia after stent implantation, resulting in increased vessel diameter, and eliminate late in stent restenosis.[9,10]

Migration and proliferation of vascular smooth muscle cells into the arterial lumen have been identified as a key mechanism of neointima formation and in-stent restenosis. Many researchers have tried to find an agent that can reduce the hyperplasia of vascular smooth muscle while do little harm to the proliferation of vascular endothelial cells.

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A study has shown that a nanoparticle drug delivery system is highly effective on this issue.[11–14] Drug-loaded nanoparticles are expected to greatly improve the drug loading per unit area due to its high specific surface area. When delivered to the lesion artery, nanoparticles can traverse vessel wall and easily internalized by smooth muscle cells due to their miniature size. Meanwhile they also act as storage vehicles to provide a sustained drug release.

In this study, we developed a novel nanoparticle coated drug-eluting stent. First, we used a novel 3D-printing technology to fabricate poly (L-lactide) (PLLA) based biodegradable stents. Then poly (D,L-lactide) (PDLLA) nanoparticles carrying sirolimus which can inhibit vascular smooth muscle cell proliferation and migration were prepared with a emulsion solvent evaporation method.[15,16] A layer of poly(vinyl pyrilidone) (PVP) or polyethylvinyl acetate (PEVA) was grafted on to the surface of PLLA stents via a low-temperature plasma polymerization method aimed at improving the adhesion between nanoparticles and the stent.[17–20] Subsequently, drug loaded nanoparticles was coated to the stents using a dip coating process.[21]

Additional experiments were carried out to evaluate the effect of drug-loaded nanoparticles on the proliferation and cytotoxicity of both human coronary artery smooth muscle cells (HCASMCs) and human coronary artery endothelial cells (HCAECs). The degradation behavior and drug release profile of the nanoparticle coated stents were also studied.

MATERIALS AND METHODS

Preparation of drug-loaded nanoparticles

The drug loaded PDLLA nanoparticles were synthesized by a modified precipitation–solvent evaporation technique.[13] Specifically, PDLLA (Leon Chemical, Shanghai, M_w: 30,000–50,000) was dissolved in a 10 mL acetone and alcohol mixture (5:5, 20 mg/mL) in a beaker. Then sirolimus (Purechem-Standard, Chengdu) was added to the PDLLA solution under stirring. A mixture of deionized water and ethanol (1:1, v/v) was added drop-wise into the polymer solution and stirred by a magnetic stirrer until turbidity was visually observed. The prepared PDLLA nanoparticle suspension was added to an aqueous solution (50 mL, 2%v/w) containing polyvinyl alcohol (Sigma, 1788, M_w: 70,000–80,000) in a beaker in a sonicator (100W, BL6-180A, Bilon). Then the mixed solution was agitated in the glass beaker using a stirrer (MS-III-Pro, Leopard Scientific Instruments) at ambient temperature for 12 h according to Ref. 22, to remove the organic solvent. PDLLA nanoparticles was collected by centrifugation (SORVALL Stratos, Thermo Scientific) at 12,000 rpm for 30 min and washed using deionized water for three times. PDLLA nanoparticles were then re-suspended in deionized water and lyophilized using a freeze dryer (ALPHA 1–4, CHRIST, Germany). The obtained PDLLA nanoparticles were stored as lyophilized powder at room temperature.

The PLLA bare stents manufactured using a proprietary 3D-printing technology was provided by Beijing Advanced Medical Technologies. In some of the tests, 3D printed single layer scaffolds (SLS) (Fig. 1) with a similar structure of the stent design were used.

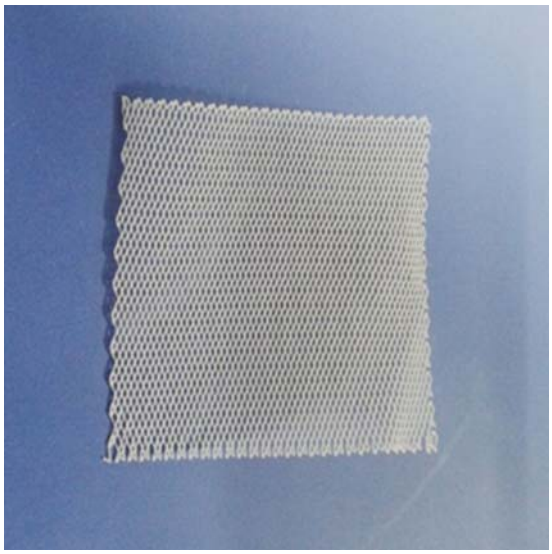

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FIGURE 1. The 3D printed single layer scaffolds (SLS) with a similar structure of the stents.

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Plasma surface polymerization

The plasma surface polymerization of PEVA and PVP to enhance the adhesion between nanoparticles and PLLA stent was carried out using a plasma surface treatment apparatus (GY-KS01, PLAUX, China) via two methods: gaseous polymerization and liquid polymerization. Both of them were performed using the same condition: gas (Ar) import 100SCCM (mL/min), discharge power supply 100 W, degree of vacuum at 25 Pa.

Method 1 *(gaseous polymerization).* The stents and SLS were put into the reactor, then 10 mL EVA or NVP was introduced into the vaporizing chamber of the plasma treatment device, vaporization temperature was set to 50°C. Then the plasma treatment was carried out under the set conditions for 30 min.

Method 2 *(liquid polymerization).* The stents and SLS were firstly soaked in EVA or NVP alcohol solution (v:v, 3:7) for 12 h at room temperature. After that the SLS was taken out from the solutions and dried under reduced pressure (40°C, 2.5 KPa, 3 h) to constant weight. Then plasma was initiated and the SLS were exposed to the air plasma for 30 min. After plasma treatment, the SLS was taken out from the chamber, washed in methanol for 30 min to remove EVA/NVP homopolymers, and finally dried under reduced pressure.

Contact Angle of Scaffold Surface

The hydrophilicity of SLS treated by plasma polymerization was characterized by the change of surface contact angles. The samples were examined using a contact angle measuring device (JC2000-C, POWEREACHR). Double distilled

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water was used and the contact angle was measured using a method of goniometry.

Coating of Stents with Nanoparticles

The plasma surface treated stents became "sticky" to drug loaded PDLLA nanoparticles. Thus the drug-loading nanoparticles were easily attached to the surface of stents. We coated the plasma treated stents by dipping them into a nanoparticle suspension in pure water with a concentration of 10 mg/mL. Then they were dried in a vacuum drying oven (40°C, 2.5 KPa, 12 h) and weighted.

The adhesion between sirolimus-loaded nanoparticles and PLLA stents was evaluated in a flowing PBS buffer (pH 7.4) in a glass beaker for 10 and 30 min under stirring using a magnetic stirrer (50 r/min). Then the stents were dried in a vacuum drying oven (40°C, 2.5 KPa, 12 h) and weighted. Stents were observed using a scanning electron microscope (SEM, Hitachi, S-4800, Japan).

Particle Size and Size Distribution

The average particle size and size distribution of the PDLLA nanoparticles were determined using a dynamic light scattering instrument (DLS) (Zetasizer Nano-ZS 90, Malvern Instruments GmbH, Herrenberg, Germany). The intensity of scattered light was detected at 90° to the incident beam. The freeze-dried powder was dispersed in ultrapure water at a concentration of 1 mg/mL using a Sonicator (BL6-180A, Bilon, 100W) for 20 min. A 500 μL suspension was used for testing each time. All the data analysis was performed in an automatic mode. Measured particle size was presented as an average value of 20 runs, with triple measurements within each run.

Drug Loading Efficiency and Drug Release

PDLLA nanoparticles of 50 mg were suspended in 10 mL methanol at room temperature for 2 h with continuous shaking to completely extract the encapsulated sirolimus. High performance liquid chromatography (HPLC) was used to determine the concentration of sirolimus in the methanol extracts. The actual drug loading efficiency E_2 was calculated as:

$$\frac{(\text{Sirolimus concentration})\times\text{Volume}}{(\text{Weight of NPs})\times(\text{Drug ratio})}\times 100\% \qquad (1)$$

The HPLC was also used to determine the sirolimus release profile in PBS (pH = 7.0). The profile used a random sampling method, and 10 mg of drug loaded PDLLA nanoparticles were suspended in 25 mL of PBS which contained Brij35 detergent (Sigma) (0.05%) and BHT (Sigma) (3 mg/L) as sirolimus stabilizer. The suspension was kept at 37°C in a shaking water bath at 50 rpm. Samples of 0.5 mL were taken at scheduled time, and were centrifuged using a filter centrifuge (SORVALL Stratos, Thermo Scientific). After centrifugation, the supernatant was analyzed by HPLC [Eurospher column (100–5C18), 250_4mminner diameter; Knauer, Berlin, Germany] and an acetonitrile/water (65/35, v/v) was used as eluent. The chromatographic conditions were as follows:50°C column temperature, isocratic, 1.0 mL/min flow rate, 278 nm UV detection, calibrated measurement range of 0.1–10.0 mg/L and a detection limit of 0.02 mg/L. The same operation was repeated for three times, and the average could be defined as the drug loading efficiency of the microspheres.

Cell Culture Study

HCASMCs and HCAECs were purchased from Cell Application (Tongpai Biotechnology). HCASMCs were expanded in DMEM (Gibco) cell growth medium supplemented with 10% fetal calf serum (FCS) (Gibco), 50 ng/mL amphotericin B and 50 mg/mL gentamicin. HCAECs were expanded in 1640 cell growth medium (Gibco) supplemented with 10% FCS, 50 ng/mL amphotericin B and 50 mg/mL gentamicin. HCAECs and HCASMCs were cultured with respective growth medium in 75 cm^2 cell culture dishes at 37°C under 5% CO_2 atmosphere until the cell density was up to 80% confluence. The cells were harvested using 0.25% trypsin and collected by centrifugation (3 min at 1000 r/min). They were then resuspended and counted using a cell counting chamber. Cells were seeded in a black 96-well plate at a density of 2000 cell per well and cultured for 24 h in preparation for the next cell viability and proliferation assays.

Cell Viability and Proliferation Assays

Cell proliferation test proceed in the Cell Counting Kit-8 (CCK-8) assays purchased from Sigma (St Louis, USA). The freshly seeded cells were incubated for 24 h in a 96-well plate. After that, the cell culture supernatant was removed from the wells and replaced by new cell culture medium suspended with sirolimus, bare NPs, sirolimus-loaded NPs. Cells were further incubated for 24 h under the same conditions. Then the culture medium in 96-well plate was replaced by fresh cell culture medium and 10 μL CCK-8 was added to each well. The cells were incubated for 4 more hours with the CCK-8 reagent to enable WSTR-8 to be reduced to formazan. Formazan fluorescence, excitation wavelength at 450 nm, was measured with a fluorimeter (Fluostar Optima, BMG, Germany). All samples were run in triplicate.

Traditional cell counting assay was also employed to accurately assess the effect of drug-eluting coating to the cell viability. SLS coated with sirolimus-loaded NPs was placed into the well, then cells in logarithmic growth phase were seeded into a 48-well plate and continued to incubate. Cell number was counted at each given time point.

Scanning Electron Microscopy (SEM)

Samples were mounted on to conductive rubber slabs and gold coated (Sputter Coater 109, Plano, Wetzlar, Germany). The gold coated samples were examined using a Hitachi S-4800 (Japan) scanning electron microscopy.

RESULTS AND DISCUSSION

Nanoparticles have much higher surface area as compared to common drug release devices, such as film and disks. PDLLA is biodegradable polymer used in many FDA

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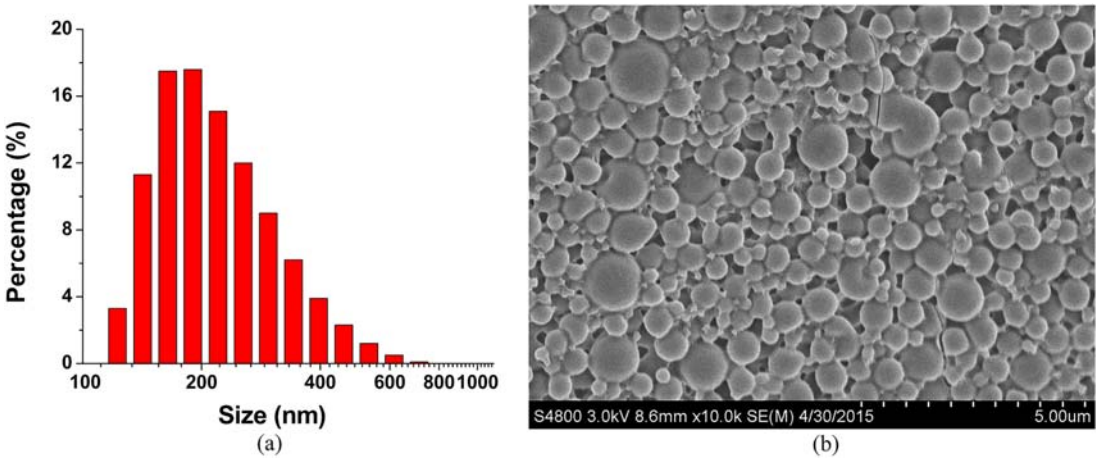

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FIGURE 2. A, B: Size and morphology of sirolimus-loaded nanoparticles. A: The particle size distribution of the sirolimus-loading nanoparticles measured by a dynamic light scattering instrument (DLS), B: the SEM image showing the surface morphology of sirolimus-loading nanoparticles.

approved implants and drug delivery devices. The precipitation–solvent evaporation method was found to be appropriate and efficient for the preparation of PDLLA nanoparticles.[23] Therefore, experiment was performed to prepare sirolimus loaded nanoparticles.

The key to apply PDLLA nanoparticles as coating to PLLA stents is to have enough adhesion between the substrate and nanoparticles. To modify the surface of the substrate to improve the adhesion of nanoparticles on the PLLA stent surfaces, a plasma surface grafting technology was employed to introduce a layer of hydrophilic polymers on to the surfaces of the PLLA. Low-temperature plasma surface grafting technology is widely used to modify surface properties of materials.[24,25] The hydrophilic polymers grafted on the surface of material in this study can enhance not only the hydrophilicity but also the adhesion of the PLLA substrate surface so that sirolimus-loaded NPs can be coated on to the surfaces of the stents via a simple dip coating process.

Drug-Eluting Stent and Nanoparticle Characterization

The size and shape of nanoparticles were determined by both dynamic light scattering (DLS) and scanning electron microscope (SEM). The hydrodynamic average particle size, as determined by DLS, is 250 ± 100 nm [Fig. 2(A)]. SEM observation showed that all nanoparticles are in sphere shape with a particle size distribution ranging from 200 to 400 nm [Fig. 2(B)].

Nanoparticles of 5, 10, and 30% of sirolimus loading were prepared for *in vitro* drug release study. The actual sirolimus content in nanoparticles was determined by HPLC, the drug loading efficiency was calculated as almost 90% according to formula (1).

The drug release study of sirolimus loaded PDLLA nanoparticles was carried out in a PBS (pH = 7.4) buffer. Due to the low stability of sirolimus in aqueous solution, BHT, which is a lipophilic antioxidant additive, combined with Brij35, a nonionic detergent, was used to increase the solubility and stability of sirolimus in aqueous PBS solution.

The drug release study was carried out by suspending10 g of nanoparticles in 25 mL PBS/detergent buffer at 37°C under constant shaking. At certain time interval, 0.5 mL of NP suspension was taken out and centrifuged at 12,000 rpm for 30 min. Then the supernatant was analyzed using a HPLC. The residual nanoparticles were resuspended in 0.5 mL fresh PBS/detergent buffer and then put back into the drug release suspension to continue the drug release study. The drug release profiles of sirolimus-loaded PDLLA nanoparticles were obtained by determining the percentage of drug released at each time point with respect to the total drug loading. Results showed that approximately 40% of the loaded sirolimus in 30% sirolimus/PDLLA NPs was released within the first 24 h, and the rest of sirolimus was released over the next 15 days (Fig. 3).

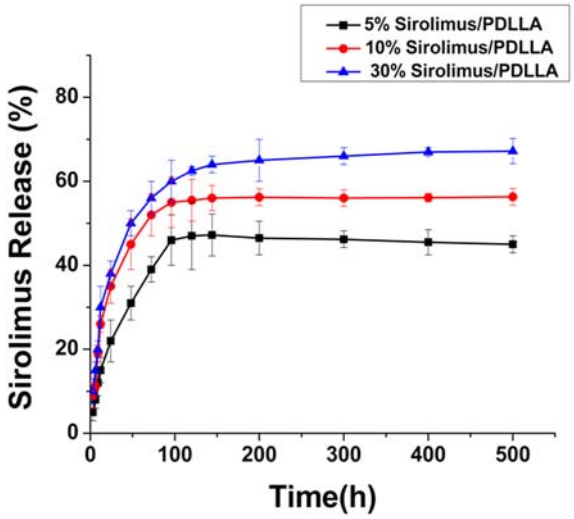

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FIGURE 3. Drug release profile of sirolimus loaded PDLLA nanoparticles.

TABLE I. Contact Angle of Stent Surface with Different Plasma Surface Treatment

Monomer	Condition			Contact Angle (θ) H_2O (°)
	Method	Power (Wf)	Time (min)	
EVA	g	100	30	50
	l	100	30	42
NVP	g	100	30	38
	l	100	30	25
Control	_	_	_	105

(g): Method of gaseous polymerization; (l): Method of liquid polymerization.

The release curve showed nanoparticles with different sirolimus content had different release characteristics. The 30% sirolimus/PDLLA NPs showed the highest rate of release than others with lower drug contents. Moreover, it also had the highest release efficiency which could reach 70%, while the 5 and 10% sirolimus/PDLLA NPs were at about 40 and 56%. This difference in drug release efficiency was likely caused by the dispersion state of drug in polymer. Higher drug content may lead to the formation of a continued drug phase channel formation and more drug could be released through these interconnected channels.

Surface Features and Surface Contact Angle of Stent Treated by Plasma

The plasma treatment was carried out via two different methods: gaseous polymerization and liquid polymerization, T1 contact angle measurement on SLS showed (Table I) that plasma polymerization of hydrophilic polymers enhanced hydrophilicity of the PLLA scaffolds greatly. Surface contact angle of the scaffolds without plasma treatment is 105°, which means the surface was highly hydrophobic. After treated by plasma grafting, the PLLA scaffold surfaces became highly hydrophilic as their surface contact angles were all greatly reduced. The contact angle could even reach 25° in the case of plasma initiated liquid NVP polymerization.

Liquid phase polymerization resulted in lower contact angle than gaseous polymerization (Table I). In the case of liquid phase polymerization, the higher degree of surface contact angle reduction was caused by the increased surface roughness resulted from etching of the surface by the liquid monomers (Fig. 4). Both EVA and NVP liquid monomers F4 worked as surface etching solvents of PLLA when the scaffolds were immersed in them. Itched surfaces had higher surface areas and the subsequently plasma grafted surfaces had an increased number of hydrophilic groups on the surface of stents. Therefore, a higher degree of surface hydrophilicity was always achieved by using plasma initiated liquid polymerization on the surface as compared to the plasma initiated gaseous surface polymerization.

Characteristics of the Novel Biodegradable Drug-Eluting Stents

Stent treated by plasma surface grafting produced highly hydrophilic surfaces. More importantly, with the surface grafting of PEVA and PVP via surface plasma polymerization, the surface of the stent becomes "sticky". Thus the drug-loaded nanoparticles could be adsorbed on to the surface of stent easily.

Since the PVP liquid-plasma polymerization produced most hydrophilic surfaces, and the EVA showed a visible stronger swelling effect on PLLA stents which in turn might

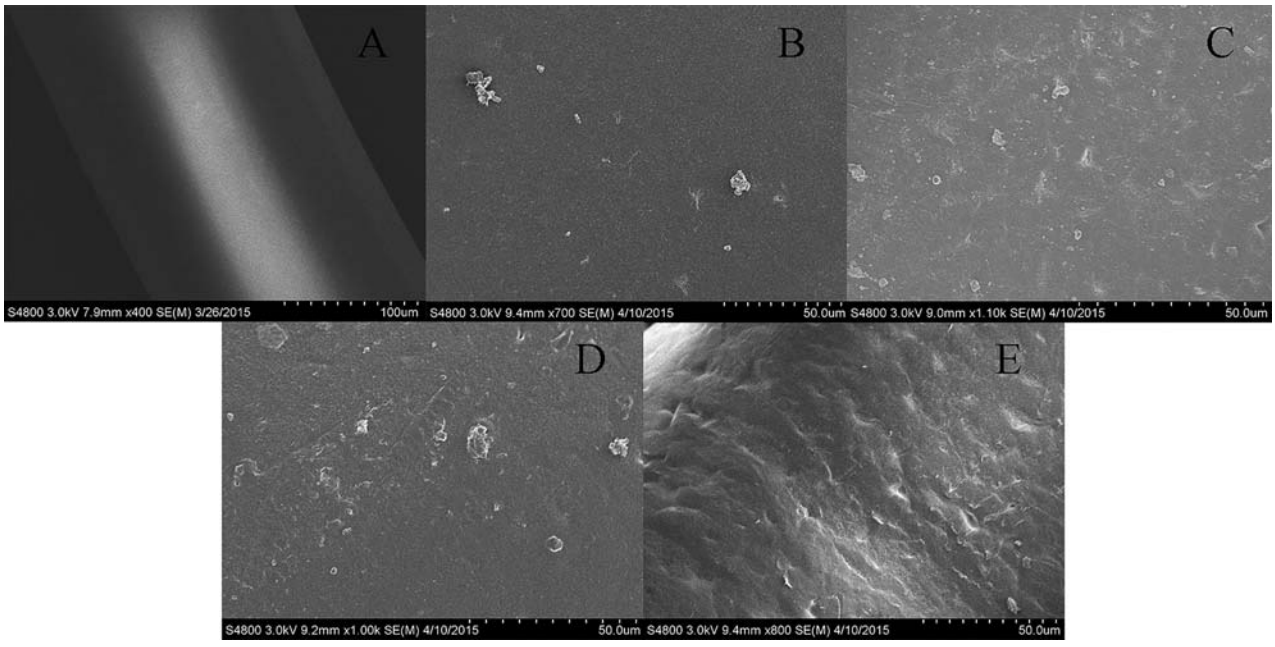

FIGURE 4. Surfaces of scaffolds with different plasma treatment. A was the bare stent used as a control; B was the scaffold treated by gaseous polymerization in NVP; C was the scaffold treated by gaseous polymerization in EVA; D was the scaffold treated by liquid polymerization in NVP; E was the scaffold treated by liquid polymerization in EVA.

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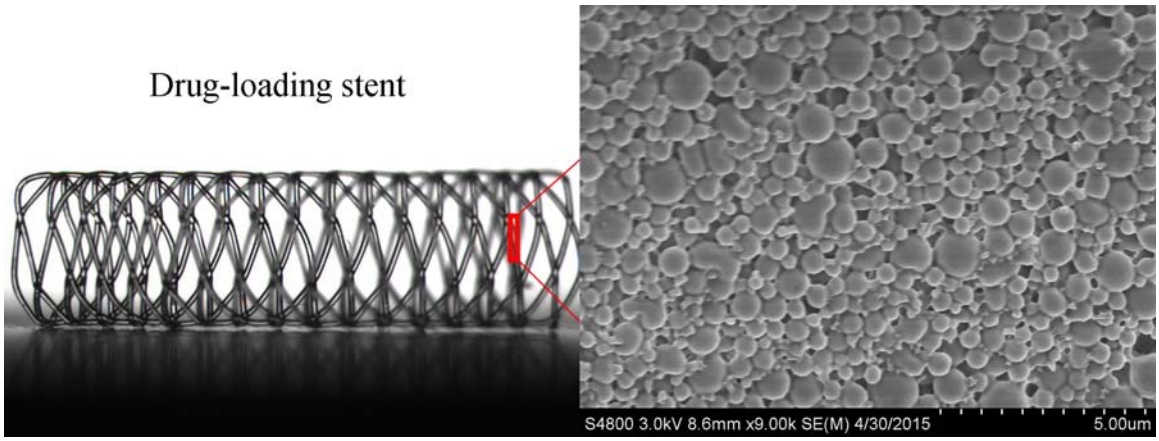

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FIGURE 5. The novel biodegradable drug-loaded eluting stent. The SEM picture showing the surface morphology was on the right.

affect the mechanical properties of the stents, therefore, only the stents treated with PVP liquid–plasma polymerization were used in the nanoparticle coating process.

The nanoparticle coating process was simply performed by dipping the plasma-treated stent into the drug-loaded NPs suspension (10 mg/mL) with deionized water, then taking out and drying in an vacuum oven (40°C, 2.5 KPa,12 h). The scanning electron microscopy observation showed that a NP coating indeed was obtained (Fig. 5).

The NP coating firmness on the surfaces of the stents was studied in a flowing PBS buffer (pH = 7.4). SEM and weight of the NPs coated stent after washing in the agitated PBS buffer for 10 min [Fig. 6(A)], and 30 min [Fig. 6(B)] showed few NPs were washed away and the weights of them barely changed.

The drug loading of the stents could be calculated with formula below:

$$\sum = (M_1 - M_2) \times E_1 \times E_2 \quad (2)$$

$M(a)$: weight of drug-loaded stent; $M(b)$:weight of bare stent; E_1: the drug ratio; E_2: the entrapment efficiency of drug-loaded NPs as determined using formula (1).

According to Table II, the sirolimus-NPs loading reached to 1.67 mg per stent compared with 0.27 mg of the bare stent and the sirolimus loading could be 450 μg calculated by formula **(2)**. When the novel biodegradable PLLA stents were delivered to the lesion sites, it is expected that the drug loaded nanoparticle coating could survive the delivery process. At the lesion sites, nanoparticles could easily enter the vascular cell through endocytosis, then sirolimus would release in the cells to prevent cell proliferation, especially for smooth muscle cells. These nanoparticle coated bioabsorbable PLLA stents could potentially achieve complete endothelialization faster while still prevent in stent restenosis efficiently.

Cell Viability or Proliferation Analysis

To investigate the influence of the novel drug loaded NP coating on cell viability and proliferation of endothelia and smooth muscle cells of blood vessels, both HCAECs and HCASMCs were incubated with 0.25 mg/mL sirolimus, 0.5 mg/mL bare PDLLA nanoparticles, 0.5 mg/mL sirolimus-loaded nanoparticles, and the SLS scaffolds coated with sirolimus-loaded nanoparticles. Cells cultured in normal condition served as a control.

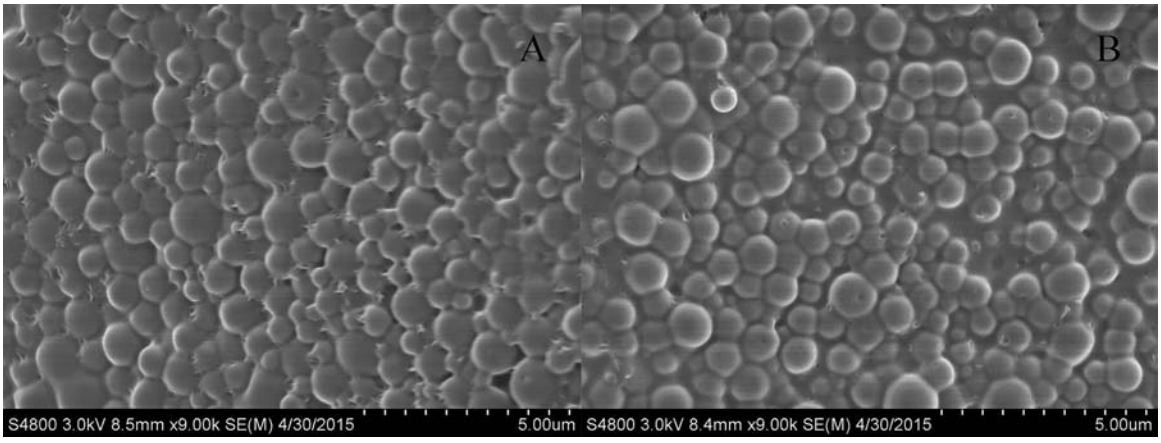

FIGURE 6. A, B: The wash off study of the sirolimus NPs coating: (A) washed for 10 min, and (B) washed for 30 min.

TABLE II. Drug Loading of the Novel Sirolimus NPs Coated Stent

Category	30% Sirolimus NPs Loading (mg)			Average NPs Loading (mg)	Average Drug Loading (μg)
	Stent 1	Stent 2	Stent 3		
Control	0.3	0.3	0.2	0.27	72
PVP(l)	1.7	1.8	1.5	1.67	450
PVP(g)	1.2	1.0	1.3	1.17	315
PVAc(l)	1.5	1.2	1.2	1.3	351
PVAc(g)	0.9	1.0	1.2	1.03	279

(g): Method of gaseous polymerization; (l): method of liquid polymerization.

These results in cell proliferation assays demonstrated that sirolimus at a concentration of 0.25 mg/mL, when presented alone in the medium, was an effective inhibitor for both HCAEC and HCASMC, and it was found (Fig. 7) that sirolimus had a little stronger inhibition effect on the proliferation of endothelial than on smooth muscle cell which has been reported by Matter et al.[26] And this might cause a delay of re-endotheliation after implantation of the sirolimus-loaded stent. This delayed re-endotheliation has been associated with an increased risk of in-stent thrombosis which had been a major issue of DES.[27,28]

As expected, plain nanoparticles showed no effect on the viability and proliferation of both HCAECs and HCASMCs (Fig. 7). Nanoparticles loaded with sirolimus, when used alone or as coating on SLS scaffolds, exhibited an inhibition effect for both types of cells, but in a different way when compared with sirolimus alone. The proliferation of HCAECs was reduced to 70% and HCASMCs was reduced to 50% for

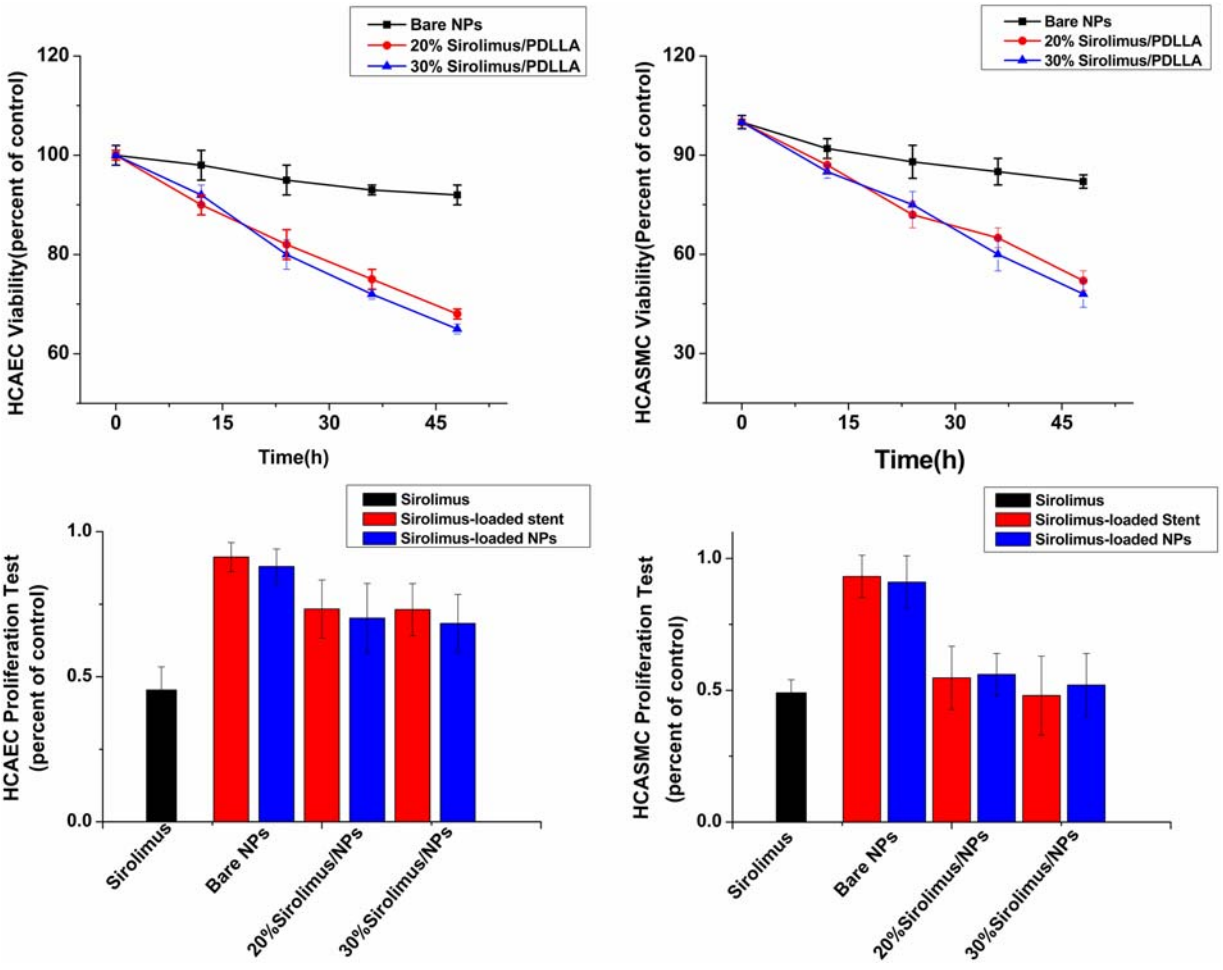

FIGURE 7. Cell viability and proliferation tests using HCAEC and HCASMC. Cells were cultured with different media in the presence of sirolimus, bare NPs, sirolimus-loaded NPs and the scaffolds coated with sirolimus loaded NPs, respectively. Cell numbers were counted at scheduled time points and compared to the control.

sirolimus-loaded PDLLA nanoparticles as compared to control. Hence, a nanoparticle coating loaded with sirolimus on a coronary stent might leave endothelial cells more viable to an extent that allows re-endotheliation of a stented vessel but still could prevent excessive smooth muscle cell proliferation. These properties of drug loaded nanoparticle coating could potentially allow a faster endothelialization while still prevent in stent restenosis resulted from overgrowth of vessel smooth muscle cells. A faster endothelialization of the implanted stents would mean a reduced risk of in-stent thrombosis and could be beneficial to the patients.

CONCLUSION

Bioresorbable PLLA stent surface grafted with PEVA and PVP could be coated with sirolimus-loaded nanoparticles efficiently. The sirolimus-loaded NPs as coating on the 3D printed PLLA stents showed pronounced inhibition effect on smooth muscle cell proliferation than on endothelial cell proliferation. Thus, this novel drug coated bioresorbable stent may prevent restenosis by inhibition of smooth muscle cell proliferation but allow a faster re-endotheliation of the implanted stents. Further study will be conducted in animals to prove the hypothesis.

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