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Surface engineering of cardiovascular stent with endothelial cell selectivity for *in vivo* re-endothelialisation

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ABSTRACT

The *in vivo* endothelialisation of materials provides a promising strategy for the rapid re-endothelialisation of a cardiovascular implantation. Although many studies have focused on improving the rapid endothelialisation through the immobilisation of bioactive molecules, it should be noted that the endothelial cells (ECs) will compete with other cell types *in vivo*. Thus, the efforts to partially enhance the EC growth without considering the cell competition might be misleading and meaningless *in vivo*. In this study, we demonstrated that the competitive growth of human umbilical vein endothelial cells (HUVECs) over human aortic smooth muscle cells (HASMCs) could be increased through the synergic action of the nonspecific resistance to phosphorylcholine and the specific recognition of the REDV peptide. Further *in vivo* data indicate that the competitive ability of ECs over SMCs, instead of the number of ECs, is a significantly more important criterion for the development of a pure endothelial layer *in vivo* and thus the attainment of a better anti-restenosis effect. Consequently, the surface tailoring of a stent to obtain high endothelial cell selectivity is likely an effective design criterion for *in situ* endothelialisation and a possible future solution for the problem of in-stent restenosis.

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1. Introduction

Cardiovascular disease is one of the leading causes of death worldwide [1,2]. Percutaneous transluminal coronary angioplasty (PTCA), which is based on stent implantation, has been an important procedure in the treatment of cardiovascular diseases, particularly coronary vascular disease [3–5]. However, in-stent restenosis (ISR) occurs when smooth muscle cells (SMCs) undergo rapid adhesion and proliferation after the stent implantation, which causes a re-narrowing of the vessel [6]. ISR is a significant concern due to its clinical application, which typically occurs in 15–20% of patients [7,8]. Although drug-eluting stents (DES) play an important role in reducing restenosis, the risks of late local endothelium regeneration events and late stent thrombotic (LAST) events are still increasing concerns for clinical long-term implantation failure [9–11]. Previous studies have demonstrated that the injury-induced migration and proliferation of SMCs and the delay of the re-endothelialisation are the major pathophysiological events that lead to neointima formation. The vessel endothelium plays an important role in the maintenance of the integrity of the vessel by preventing thrombosis and hyperplasia. A rapid re-endothelialisation has been proven to provide a potential technique to prevent ISR and LAST [5,12,13].

Several researchers have successfully enhanced endothelial cell adhesion and proliferation by immobilising extracellular matrix (ECM) molecules or synthetic cell-adhesive peptides on material surfaces [14–16]. However, very little attention has been paid to SMCs [17,18]. The unfortunate reality is that the ECs compete with many different cells *in vivo* [19–24]. Thus, the effort to partially enhance the growth of EC without considering the cell competition might be meaningless and misleading *in vivo* [25,26]. A number of researchers [5,17,18], including our group [5], have recently demonstrated that the immobilisation of an EC-specific ligand onto a cyto-compatible matrix can specifically promote EC adhesion and rapid *in situ* endothelialisation. Although most efforts to obtain high selectivity have focused on using the highest possible density of the specific ligand, the natural systems function differently because most highly selective complexes in biological systems are achieved through a low ligand density. The fluid mosaic structure of cell membranes, for example, is constructed by a phospholipid bilayer with embedded proteins and carbohydrates. Because there are a large number of different ligands, each of which has a different biological function, the density of one single type of protein or carbohydrate is quite low. The high selectivity in a complex biological system is achieved by the synergic action of the nonspecific resistance to phosphorylcholine (PC) [27–31] and the specific recognition

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of biomolecules. The Arg–Glu–Asp–Val (REDV) peptide is an EC-specific ligand that mediates the adsorption and migration of ECs via the integrin $\alpha_4\beta_1$ subunit, but does not allow the adhesion of SMCs [18,32] and platelets [33]. In this study, we hypothesised that the combination of nonspecific resistance to PC and the EC-preferential ligand will enhance the competitive adhesion of ECs over SMCs. Thus, the REDV peptide was immobilised onto poly (*n*-stearyl methacrylate (SMA)-*co*-*p*-nitrophenyloxycarbonyl poly(ethylene glycol) (MEONP)) (PSN) and poly(2-methacryloyloxyethyl phosphorylcholine (MPC)-*co*-*n*-stearyl methacrylate (SMA)-*co*-*p*-nitrophenyloxycarbonyl poly(ethylene glycol) methacrylate (MEONP)) (PMSN). The adhesion and proliferation of human vascular ECs and SMCs on the bio-inspired coating were examined to investigate the synergistic action of the PC and the REDV peptide. A co-culture system and cell migration assays were also used to evaluate the rapid competitive adhesion and migration of ECs and SMCs. An *in vivo* study was conducted using a rabbit iliac artery injury model to evaluate the *in situ* re-endothelialisation and the anti-restenosis effects of the modified stents. The correlation between the competitive ability of ECs over SMCs and *in situ* re-endothelialisation is discussed.

2. Materials and methods

2.1. Materials

Poly(ethylene glycol) methacrylate (PEGMA, $M_n = 360$) and stearyl methacrylate (SMA) were commercially obtained from Aldrich Chemical Co. and used without further purification. The average number of oxyethylene repeating units in PEGMA is 6. p-Nitrophenyl chloroformate was purchased from Darui Chemical Co. (Shanghai, China). α,α'-Azobisisobutyronitrile (AIBN) was re-crystallised from methanol. Chloroform and triethylamine (TEA) were purchased from Shuanglin Chemical Co. and purified through distillation under reduced pressure. The Arg–Glu–Asp–Val (REDV) peptide was obtained from Shanghai Science Peptide Biological Technology Co. Fluorescein diacetate (FDA) was obtained from Sigma–Aldrich. The orange [5-(and 6)-(((4-chloromethyl)-benzoyl)-amino)-tetramethyl-rhodamine, (CMTMR)] cell-tracker dye was purchased from Molecular Probes. The Griess reagent nitric oxide assay kit was obtained from Beyotime Biotech (Jiangsu, China). Human umbilical vein endothelial cells (HUVECs) were kindly provided by the Second Affiliated Hospital of the Medical College of Zhejiang University, and human aortic smooth muscle cells (HASMCs) were purchased from Beifang Weiye Development Co. (Shanghai, China).

2.2. Synthesis of MEONP and PMSN

MPC and MEONP were synthesised using a previously reported method [34–36]. Briefly, MEONP was synthesised according to the following procedure: 9.8 mL of PEGMA and 4.2 mL of TEA were placed in a four-necked round-bottomed flask with a magnetic stirrer and a thermometer; 20 mL of chloroform was then added to the mixture. After the solution was cooled at −20 °C, p-nitrophenyl chloroformate (6.05 g) dissolved in 15 mL of chloroform was added dropwise to the stirred solution over a period of 1 h. The temperature of the reaction mixture was maintained at −20 °C for 4 h. After the precipitate (triethylamine hydrochloride (TEAC)) formed in the reaction mixture was filtered off, a yellow solution was obtained. The solution in the filtrate was evaporated under reduced pressure. After the addition of dry diethyl ether to the yellow solution, the filtration and evaporation steps were repeated twice. MEONP was then obtained as a yellow oily liquid. The structure of MEONP was confirmed by ^{1}H NMR.

PMSN (poly(2-methacryloyloxyethyl phosphorylcholine (MPC)-*co*-*n*-stearyl methacrylate (SMA)-*co*-*p*-nitrophenyloxycarbonyl poly(ethylene glycol) methacrylate)) was synthesised through the conventional radical polymerisation of MPC, SMA, and MEONP using AIBN as an initiator. The polymerisation was performed in a polymerisation tube at 60 °C for approximately 24 h. After polymerisation, the reaction mixture was precipitated using an excess amount of dry methanol as the solvent; this step was repeated twice. The ratio of the monomer unit compositions in PMSN was determined by ^{1}H NMR, XPS, and the UV spectrum (268 nm). As reference samples, PMS (2-methacryloyloxyethyl phosphorylcholine-*co*-*n*-stearyl methacrylate) and PSN (poly (*n*-stearyl methacrylate (SMA)-*co*-*p*-nitrophenyloxycarbonyl poly(ethylene glycol))) were synthesised using the same procedure.

2.3. Conjugation and immobilisation of the REDV peptide

Polyethylene terephthalate (PET) sheets were used as the substrates for polymer coating. After PSN and PMSN were completely dissolved in tetrahydrofuran (THF), the dip-coating step was performed. The substrates were vertically dipped into the solution and removed at a constant speed. The process was repeated six times, and the sample was then placed into a vacuum oven overnight. The substrates were then dipped into the REDV solution (phosphate buffer solution pH 7.4, 200 μg/mL) and allowed to react with the active esters of PMSN at 4 °C for 24 h. BSA was used as the blocking reagent [34]. BSA in PBS solution (pH 7.4, 200 μg/mL) was added into the separate wells with the polymer coatings. After incubation for 24 h at 4 °C, the wells were washed three times with PBS. The surface analysis was performed using the UV spectrum (400 nm).

2.4. Surface morphology of polymer-coated stents

The 316L stainless steel coronary stents (bare metal stents, BMS), which were 15-mm long and had a 2-mm diameter (Antai Tech Co., Ltd., Beijing, China), were immersed in an alcohol/water (1/1 v/v) solution for 4 h to remove any oily dirt and were then washed with an excess amount of deionised water. The stents were dried under reduced pressure at 30 °C for 24 h.

The PSN–REDV- and PMSN–REDV-modified stents were prepared using the ultrasonic atomisation spraying method, as previously reported [37]. PSN or PMSN was dissolved in THF to obtain a 5 mg/mL spraying solution. The solution was then sprayed onto the stent surface. The prepared PMSN stents were maintained in a vacuum for 24 h at room temperature to remove all residual solvent molecules. The immobilisation of the REDV peptide onto the polymer-coated stent was performed using the abovementioned method that was used with the PET substrates.

The surface morphology of the polymer-coated stents was examined through scanning electron microscopy (SEM) before balloon expansion. The stents were then mounted onto an angioplasty balloon (2.0 mm), and the balloon was inflated to a maximum pressure of 12 atm for 30 s, deflated and withdrawn slowly. The post-expansion stents were also examined using SEM.

2.5. Cell culture

HUVECs and HASMCs were cultured in 25-cm^2 cell culture flasks at 37 °C in a humidified atmosphere with 5% CO_2 and 95% air. The HUVECs were incubated in RPMI-1640 medium (Gibco) supplemented with 10% foetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin. The HASMCs were cultured in DMEM medium with 4.5 g/L glucose. The cultures were maintained at 70–80% confluence through subculturing using standard techniques. All of the non-attached cells were removed by washing with PBS. The cell density for the experiments was determined using a hematocytometer. The cells were plated in 96-well tissue culture polystyrene (TCPS) plates at a cell density of 5000 cells per well of each cell type.

The cell adhesion and growth was determined using the FDA assay. Briefly, fluorescein diacetate (FDA, Sigma) was dissolved in acetone at a concentration of 5 mg/mL. The adherent cells were stained with fluorescein diacetate (FDA) and visualised by fluorescent microscopy (Zeiss, Germany). The fluorescent images were acquired using an Olympus DP71 microscope. The cells were seeded onto PET films and 96-well TCPS plates with different polymer coatings. At predetermined time points (4 h, 24 h, and 48 h), the adherent cells were stained with FDA and visualised by fluorescent microscopy. The fluorescent images were acquired using an Olympus DP71 microscope at a 20× magnification with a fluorescein filter (excitation at 488 nm). All of the experiments were performed at least twice. Three replicates of each sample were prepared, and 10 random images were acquired. The number of cells in each image was determined through manual counting, and the results were averaged. The average number of adhered cells from three replicates was then converted to the cell adhesion density.

The cell morphology on different surfaces was analysed by SEM to investigate the adhesion of the cells to the different samples. First, the cells were seeded onto PET films and 24-well TCPS plates with a PMSN–REDV surface. After a 72-h culture, the surfaces were rinsed twice with PBS for 10 min and then soaked in 2.5% glutaraldehyde for 2 h at 4 °C. After rinsing with tri-distilled water three times, the samples were dehydrated in an ethanol gradient with increasing concentrations of ethanol (30–100%) for 5 min at each concentration and allowed to dry in a desiccator at room temperature. Finally, the samples were gold-coated using a sputter-coater and analysed by SEM.

The nitric oxide (NO) that is produced by endothelial cells is an important physiological parameter. The amount of nitrite, which is an NO derivative, was determined using the Griess reaction. The nitrite detection kit was used according to the instructions provided by the manufacturer (Beyotime Institute of Biotechnology, China). Briefly, the cells were separately seeded on PET film, PMS film and 96-well TCPS plates with a PMSN–REDV surface. After 3 days of culture, the supernatants of each well were collected, and the relative NO content in the supernatants was measured. A volume of 50 μL of the supernatant or the standard ($NaNO_2$) was mixed with 50 μL of Griess Reagent I and 50 μL of Griess Reagent II in a 96-well plate. After the mixture was allowed to react in the dark for 15 min, the release of NO was read in a microplate spectrophotometer at 490 nm [38]. Three replicates were read for each sample, the mean value of the three replicates was used as the final result.

2.6. HUVECs and HASMCs co-culture

The co-culture of HUVECs and HASMCs was performed according to the protocol described by Murthy SK et al. [32]. The HUVECs and HASMCs were incubated in

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