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The effect of a layer-by-layer chitosan–heparin coating on the endothelialization and coagulation properties of a coronary stent system

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ABSTRACT

A biomacromolecular layer-by-layer coating process of chitosan/heparin onto a coronary stent is designed for the acceleration of the re-endothelialization and healing process after coronary stent deployment. The results of *in vitro* culturing of porcine iliac artery endothelial cells as well as the measurements of the APTT, PT and TT supported the rationale that the combination of chitosan and heparin could bring both endothelial cell compatibility and haemocompatibility to the stent surface. A porcine coronary injury model and arteriovenous shunt model were used for the further evaluation of the application of this kind of surface-modified stainless steel stent *in vivo*. The final results proved that this facile coating approach could significantly promote re-endothelialization and was safer compared with bare metal stents for its much improved anticoagulation property.

1. Introduction

The introduction of drug-eluting stent (DES) systems has already been proved to be a landmark in coronary intervention therapy. They successfully suppress the restenosis rate to below 10% in selected cases [1,2]. Consequently they soon became a hot spot in the field of intervention therapy. However, some recent reports indicate that DES has induced some complications such as stent thrombosis, an allergic response to the polymers, poor attachment between stent and arterial wall, and even worse, aneurysms [3–6]. Among all of these, the in-stent thrombosis, especially Late Angiographic Stent Thrombosis (LAST) was the most serious complication of DES [4–6] because most of the affected patients died or suffered a myocardial infarction, in spite of its low incidence [4–6].

In both animal [7–9] and human [10–12] studies, whether it was Cypher® or Taxus®, which are the two type of DESs most widely used in clinical therapies nowadays, a homologous pathologic phenomena was found, namely that endothelialization and healing on the stent surface were delayed [13]. This is supposed to be an important trigger for LAST. This side effect is partly blamed on the drug loaded in the DES (Rapamycin in the case of Cypher® and paclitaxel in the case of Taxus®). Another reason for the delay of endothelialization and the formation of thromboses maybe ascribed to the polymer coating on the stent surface.

Thus, there is a great demand for a new generation of coronary stent systems aiming at rapid re-endothelialization on the stent surface, which could provide protection against thrombus as well as minimizing restenosis [14,15]. Different approaches have been attempted to accelerate the surface endothelialization of the stent. Shirota et al. fabricated an intravascular stent seeded by endothelial progenitor cells (EPCs) and tested it *in vitro* [16]. This showed potential application as a new therapeutic device for re-endothelialization or endothelium lining or paving at an atherosclerotic arterial wall. Coronary stents coated with anti-CD34 antibodies, which capture a patient's EPCs to accelerate the natural healing process were also reported [17] and recently commercialized by OrbusNeich Ltd., where the EPCs circulating in the host bloodstream were involved in the repair of blood vessel walls. However, the use of biologically active substances on the stents may make production and sterilization processes difficult. Another limitation may lay in the relatively low content of the EPCs in blood.

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In the present study, we tried to achieve rapid healing of endothelium on the stent surface in a simpler way by coating the stent surface with a cytophilic natural polymer layer. Chitosan and heparin were chosen for their oppositely charged polymer electrolyte characteristic to form a stent coating through a layer-by-layer (LBL) self-assembly method. LBL self-assembly technique is a relatively new and easy approach to the preparation of complex polymer membranes, which takes advantage of the static or hydrogen-bond interactions between different kinds of macromolecules. This kind of membrane, although very thin, retains the original properties of the component polymers [18–20].

Chitosan (CS) is now widely studied and used as a matrix for tissue engineering and drug release because of its good biocompatibility and biodegradability. It has been reported that chitosan plays a critical role in cell attachment and growth [21–23]. The second material, heparin, (HEP) is the most commonly used anticoagulant reagent in clinical use. It has an incontrovertible effect on suppressing sub-acute in-stent thrombus [24–26]. It has already been proved that the CS/HEP LBL coating is anti-adhesive and antibacterial [27,28].

In this paper, CS/HEP, which could be efficient in promoting the re-endothelialization process, was chosen for the surface layer of the stent in order to reach a balance between endothelial cell affinity and thrombus resistance in the coronary stent system after stent implantation. The endothelial cell compatibility of the CS/HEP LBL coating was evaluated both *in vitro* and *in vivo* with a porcine coronary injury model. Its haemocompatibility was also estimated both *in vitro* and *in vivo*.

2. Experiments

2.1. Preparation of the chitosan/heparin LBL stents

316L stainless steel coronary stents (bare metal stents, BMS) 18 mm long and 2.5–3 mm in diameter (From LifeTech Scientific Co, Ltd., Shenzhen, China) were immersed in alcohol/water (1/1 v/v) solution for 4 h to remove oily dirt, and were then washed with a large amount of deionized water. They were dried under reduced pressure at 30 °C for 24 h.

The cleaned BMS were subsequently immersed in a 0.1 wt% ethanol solution of 3-aminopropyltriethoxysilane (APTES, purchased from ACROS Organics, Belgium) for 4 h at 37 °C, then rinsed with deionized water and dried in air at 50 °C.

The aminolyzed stents were treated with 0.01 M HCl solution for 2–3 h at room temperature and washed with a large amount of water. They were then dipped in the 0.1 wt% heparin sodium salt (HEP, Research Chemicals Ltd.) aqueous solution for 15 min and subsequently rinsed with pure water. The heparinized stents were then placed for 15 min into the solution made with 0.2 wt% acetic acid and 0.1 wt% chitosan (CS, Purchased from Sigma) with a minimum deacetylation degree of 85%, followed by the same rinsing procedures. Several bilayers of CS and HEP were prepared by repeating the deposition process mentioned above, to produce a stable supramolecular complex film by electrostatic interaction. Finally, the samples were dried at 30 °C under reduced pressure for 48 h.

2.2. Quartz crystal measurement of the chitosan/heparin LBL membrane

A KSV QCM-Z500 instrument was used to monitor the LBL multilayer buildup. In situ dissipative quartz crystal measurement (QCM) analysis mode was employed as reported [29]. A silicon–gold-coated quartz crystal was initially immersed in H_2SO_4/H_2O_2 (7/3, v/v) solution for 10 min for surface cleaning, then washed with a copious amounts of deionized water, and dried under nitrogen. It was subsequently immersed in a 0.1 wt% ethanol solution of APTES for 4 h at 37 °C, then rinsed with deionized water and dried under nitrogen. The surface aminolyzed silicon–gold-coated quartz crystal was settled in the QCM chamber and deionized water was injected as a buffer for equilibrium. HEP aqueous solution of 0.1 wt% was injected at 100 μl/min continuously until the QCM traces did not vary. Buffer was pumped in again and 0.1 wt% CS dissolved in 0.2 wt% acetic acid was injected thereafter at the same speed for the next equilibrium. HEP and CS were then alternately injected into the chamber for the buildup of LBL multilayers on the quartz crystal surface. Frequency shift vs. time (Δf–t) curves were recorded to monitor the stability of the adsorbed CS/HEP multilayers. All measurements were performed at 25.0 °C.

2.3. Cell culture and migration assay

Porcine iliac artery endothelial cells (PIEC, purchased from the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences) were cultured in 316L 35 mm diameter stainless steel dishes, which were treated with the same LBL technique described above, for the evaluation of cell affinity for the CS/HEP LBL coating. Untreated 316L stainless steel dishes were used as a control. PIECs were grown in TCPS flasks with Dulbecco's Modified Eagle's Medium (DMEM, HYCLONE Ltd.), supplemented with 20% fetal bovine serum, 300 μg/ml L-glutamine, 100 IU/ml penicillin and 25 μg/ml streptomycin solution at 37 °C in a humidified atmosphere containing 5% CO_2.

2.4. In vitro endothelial cell proliferation

PIECs at the logarithmic phase were cultured at 5×10^4/ml in the 35 mm diameter 316L stainless steel dishes, which were treated with the same LBL technique ($n = 6$), and in the untreated 316L stainless steel dishes ($n = 6$) as well. These PIECs were cultured for 24 h in DMEM. General viability of cultured cells was assayed at 450 nm with a Cell Counting Kit-8 (Dojindo Laboratories, Tokyo, Japan).

2.5. In vitro endothelial cell migration

PIECs on the logarithmic phase were cultured at 5×10^4/ml on the LBL treated 316L stainless steel dishes with 35 mm diameters ($n = 6$) as well as on the untreated ones ($n = 6$), until the buttons of all the samples were fully covered with PIECs. A sterile scraper was used to carefully scrape off the cells along the midline of the dishes while being viewed under a Nikon TE200 inverted microscope. Then the remaining cells on the samples were incubated at 37 °C. Time-lapse phase contrast images were captured every 30 min using the Nikon TE200 inverted microscope combined with a Metamorph program. Cells were continuously observed and counted for 72 h.

2.6. In vitro haemocompatibility evaluation

The 316L stainless steel dishes treated with the same LBL technique were also used in the evaluation of the *in vitro* haemocompatibility of the CS/HEP LBL surface coating, for convenience. Human blood serum obtained from healthy volunteers was mixed with 0.2 ml trisodium citrate (109 mmol/l) and transferred into 316L stainless steel dishes coated with CS/HEP LBL membranes, for a contact time of 1 h. The untreated 316L stainless steel dishes were used as a control. Plasma was prepared by centrifuging the treated blood at 3000 rpm for 15 min. Then full human plasma activated partial thromboplastin time (APTT), prothrombin time (PT) and thromboplastin time (TT) of the samples were measured on an automatic Sysmex CA-1500 using DADE BEHRING Actin, DADE BEHRING Thromburel's and DADE BEHRING Test-Thrombin Reagents. The paired t-test was used and the significance level was set to $p < 0.03$.

For a further investigation of the stability of the haemocompatibility of the CS/HEP LBL membranes, both treated and untreated stainless steel dishes were soaked in a phosphate buffered solution (PBS, pH = 7.4) for 1day ($n = 5$), 3 days ($n = 5$), 7 days ($n = 5$) and 14 days ($n = 5$), respectively. Their haemocompatibilities were evaluated thereafter.

2.7. Animal experiments

All animal experiments were approved by the Animal Care and Use Committee of Fudan University. Experiments were performed in healthy GuangXi male pigs (30 kg, obtained from the Shanghai Animal Administration Center). Starting from three days before the procedure and throughout the follow up period, all animals received 300 mg Aspirin and 250 mg Ticlopidine daily by mouth, fed with normal forage. After an overnight fast the animals were sedated with 5 mg/kg Ketamine hydrochloride and 0.5 g/kg Diazepam. Anaesthesia was induced by 25 mg/kg sodium pentobarbital and following endotracheal intubation, the pigs were connected to a ventilator that administered a mixture of oxygen and nitrous oxide (1:2, v/v).

2.8. Arteriovenous shunt model

The arteriovenous shunt model was employed as reported [30] for the semi-*in vivo* evaluations of the haemocompatibility of CS/HEP coated stents. Under sterile conditions, both carotid and femoral arteries of the pig were isolated and side branches were clipped and cut. A disinfected 30 cm × 3 mm extracorporeal circulation pipe with valve, wherein the stents were placed and expanded, was connected as a shunt system to complete the circuit between the arterial and venous circulation. The total flow rate through the pipe was controlled at 90 ml/min. After a blood flush in the arteriovenous shunt system for 1 h, the pipe was cut open, both of the CS/HEP coated stents ($n = 5$) and BMS ($n = 5$) were taken out of the pipe and carefully rinsed with PBS. Then all the samples were freeze dried and the thrombin weights were measured and compared.