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Biomimetic apatite formed on cobalt-chromium alloy: A polymer-free carrier for drug eluting stent

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ABSTRACT

In this study, sirolimus (SRL) was loaded within biomimetic apatite formed on cobalt-chromium (Co-Cr) alloy, which has been reported for the first time, to inhibit the in-stent restenosis. Two different groups of loading SRL within biomimetic apatite were prepared: Group A (mono-layer of apatite/SRL) and Group B (bi-layer of apatite/SRL). Group A and Group B showed the biphasic pattern of SRL release up to 40 and 90 days, respectively. The attachment of human artery smooth muscle cell (HASMC) for both Group A and Group B was significantly inhibited, and proliferation dramatically decreased with the release of SRL. Noteworthily, biomimetic apatite alone also suppressed the SMC proliferation. The porous biomimetic apatite uniformly covered Co-Cr stent without crack or webbings. After balloon expansion, the integrity of biomimetic apatite was sufficient to resist delamination or destruction. Thus, this study demonstrated that biomimetic apatite is a promising drug carrier for potential use in stents.

1. Introduction

A drug-eluting stent (DES) emplacement following percutaneous transluminal coronary angioplasty (PTCA) is one of well-established treatment techniques for occlusive blood vessel [1,2]. A DES is commonly a metallic platform coated with drug-loaded non-degradable/degradable polymers [3,4]. The polymer coating contained drug, such as sirolimus (SRL) or paclitaxel (PAT), has been successful in inhibition of thrombus formation, inflammation, and vascular smooth muscle cell (VSMC) proliferation [5,6]. However, recent concerns regarding the mid- to long-term safety of these polymer-based DES have been raised [7,8], mostly because of the late and very late in-stent restenosis, partially attributed to the presence of polymer coating [9,10].

As a consequence, there is a huge demand in improving the safety and efficacy of DES with biocompatible polymer-free coating and offering controlled eluting of drugs over two months [11,12]. For example, a DES has been developed with the combination of a nickel-titanium platform and a porous TiO_2 surface coating, which was proved feasible and safe [13]. Mani et al. delivered PAT from cobalt-chromium (Co-Cr) alloy surfaces without using any carriers, and a sustained PAT release was presented for up to 56 days [14].

From a biomedical point of view, apatite can be another promising alternative to polymer matrices. As a naturally occurring inorganic material, apatite has excellent biocompatibility and high affinity to many bioactive molecules [15,16]. Comprehensive studies on bone regeneration, both *in vitro* and *in vivo*, have demonstrated that apatite coated implants show no evidence of toxicity, and more biocompatible than other commonly used polymeric coatings [17,18]. The apatite coatings gradually degrade over 4–12 months *in vitro* and *in vivo*, and are completely dissolved [19]. Furthermore, apatite can maintain the survival of endothelial cells without any cytotoxic effect and proinflammatory phenotypes [20]. Although apatite coating has been commonly applied to improve surface biofunctionality for orthopedic and dental implants [21,22], few studies have been reported on DES with apatite, except the clinical trial results of polymer-free hydroxyapatite SRL-eluting stent reported by Costa et al. [19,23,24].

In our previous studies, fibronectin and osteogenic growth peptide were efficiently loaded within biomimetic apatite formed

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on titanium [25–28]. The molecule loading methods, adsorption and/or coprecipitation, were successfully employed to regulate the release kinetics of loaded compounds [27]. In this study, Co-Cr alloy was chosen as the stent platform, which can offer ultra-thin struts due to its high mechanical strength [14]. Although forming biomimetic apatite has been proved feasible on metallic materials, limited literature reports the formation of biomimetic apatite on Co-Cr substrates. Present authors, for the first time, reported a two-step chemical treatment to activate the surfaces of Co-Cr and induced the formation of biomimetic apatite on Co-Cr substrates [29]. The two-step treatment is etching Co-Cr substrates with the mixture of HNO_3, HF and H_2O_2 firstly, and then incubating in a NaOH solution. When immersing such activated Co-Cr in a metastable Dulbecco's phosphate-buffered saline (DPBS) containing $CaCl_2$, biomimetic apatite is simply formed.

In the present study, SRL, which shows anti-migration and anti-proliferation effects on human artery smooth muscle cell (HASMC) [30], was loaded within biomimetic apatite. *In vitro* physico-chemical characteristics, drug release profiles were investigated. HASMCs were used to evaluate the cellular responses to SRL-loaded biomimetic apatite *in vitro*. The initial cell attachment, morphology and cell proliferation were examined.

2. Materials and methods

2.1. Preparation of activated Co-Cr alloy

The discs of Co-Cr alloy (L605, 10 mm in diameter, and 2 mm in thickness) were used. The specimens were cleaned by ultrasonication in acetone, pure ethanol, and distilled water for 15 min each, and then were dried using N_2 gas. The cleaned Co-Cr substrate was etched in a mixture of 10 ml 70 wt% HNO_3, 10 ml 48 wt% HF and 10 ml 30 wt% H_2O_2 for 1 h in ultrasonic bath. The specimen was gently washed with distilled water, and dried by N_2 gas. The acid etched Co-Cr was then soaked in 5 ml 1 M NaOH solution at 140 °C for 6 h, washed gently with distilled water, and dried at 40 °C. These chemical treated Co-Cr alloy specimens are referred to hereafter as activated Co-Cr.

2.2. Solutions and SRL used

Reagent grade $CaCl_2$ (100 mg/L) was dissolved in DPBS (calcium/magnesium free, Thermo Fisher, USA) to prepare the metastable DPBS (mDPBS) solution. SRL was dissolved in dichloromethane (DCM, 100 mg/ml) as the stocking solution, and diluted in 100% ethanol (1 mg/ml) to prepare the working solution. The mDPBS was sterilized by filtration using a membrane with a pore size of 0.2 μm before use.

2.3. Loading SRL with biomimetic apatite on activated Co-Cr

Fig. 1 shows two ways of loading SRL within biomimetic apatite. Each activated Co-Cr was sterilized in 75% ethanol, distilled water, and then placed under UV light for 30 min. The sterilized samples were firstly immersed in 5 ml mDPBS at 37 °C for 12 h to biomimetically deposit apatite. For Group A (mono-layer of apatite/SRL), a 100 μl aliquot of the SRL working solution was pipetted on the surface of each biomimetic apatite coated Co-Cr using a micropipette in clean bench. The ethanol was allowed to evaporate leaving behind a residue of SRL within biomimetic apatite. Group B (bi-layer of apatite/SRL) was prepared by re-immersing Group A into newly prepared 5 ml mDPBS for 12 h to form the second layer of biomimetic apatite, and then dropping another 100 μl SRL working solution onto the second biomimetic apatite.

2.4. Surface characterization

The surfaces of activated Co-Cr, biomimetic coated Co-Cr, Group A and Group B were characterized using field emission scanning electron microscopy (FSEM, JSM-6500F, JEOL, Japan), attenuated total reflection-Fourier transform infrared spectroscopy (ATR-FTIR, Nicolet 5700, Thermo Electron, USA), and thin-film X-ray diffraction (XRD, Rigaku Corporation, Japan) using Cu-Kα radiation over the 2θ range 10–80° with a step size of 0.01°.

2.5. In vitro *drug release test*

For the measurement of total amounts of loaded SRL, samples (n = 5) of Group A or Group B were ultrasonically incubated in a mixture of phosphate-buffered saline solution (PBS, pH 7.4, Thermo Fisher, USA) and DCM to dissolve all drugs in solution. The amounts of SRL in PBS/DMC mixture were determined using high-performance liquid chromatograph (HPLC, UltiMate 3000, Thermo Fisher, USA). The concentration of SRL was calculated from the standard calibration curve of SRL solution.

For an *in vitro* drug release test, samples (n = 5) of Group A or Group B were incubated in 2 ml of PBS at 37 °C for various times up to 90 days. At each predetermined period, the supernatant was completely collected and 2 ml of fresh PBS was added. The released SRL in the collected supernatants was analyzed using HPLC. The surface morphologies of samples before and after drug release were observed using FESEM.

2.6. HASMC culture

HASMCs (ATCC, USA) were thawed and cultured in a complete Dulbecco's Modified Eagle's medium (DMEM, Hyclone, USA), supplemented with 10% fetal bovine serum (FBS, Hyclone, USA) and 1% antibiotic antimycotic solution (Thermo Fisher, USA) at 37 °C and 5% CO_2 in humidified environment. Studies were performed with HASMCs between the third to seventh passages. The growth medium was changed every 3 days until the cells reached 80–100% confluence.

2.7. Attachment of HASMCs

HASMCs were seeded on the samples of activated Co-Cr, biomimetic apatite coated Co-Cr, Group A and Group B (n = 5) at a cell density of 4×10^5 cells/ml and allowed to attach for 4 h in growth medium without serum. Cell attachment was performed using Cell Counting Kit-8 (CCK-8, Beyotime, China) according to the manufacturer's protocol. After 4 h incubation, samples were rinsed with PBS, and then 250 μl of fresh growth medium with 25 μl of CCK-8 reagents were added to each sample. The cell culture plates were incubated under the same cultivation conditions for another hour, and then reagents were carefully transferred to 96-well plates. The absorbance was measured using a microplate reader (ELx808; BioTek Instrusments, USA) at 450 nm. To observe the morphology of adherent cells on the samples, cells were fixed with 10% neutral buffered formalin (Sigma, USA). The cells were then permeabilized using 0.5% Triton X-100 (Beyotime, China). Subsequently, the cytoskeleton was stained by rhodamine-conjugated phalloidin (Thermo Fisher Scientific, USA) for 30 min followed by counter-staining with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma, USA) to visualize the nuclei. Finally, the fluorescent images were taken through a confocal microscope (Nikon C2, Japan) in random field.

2.8. Proliferation of HASMCs

HASMCs were seeded on the samples of activated Co-Cr, biomimetic apatite coated Co-Cr, Group A and Group B (n = 5) at